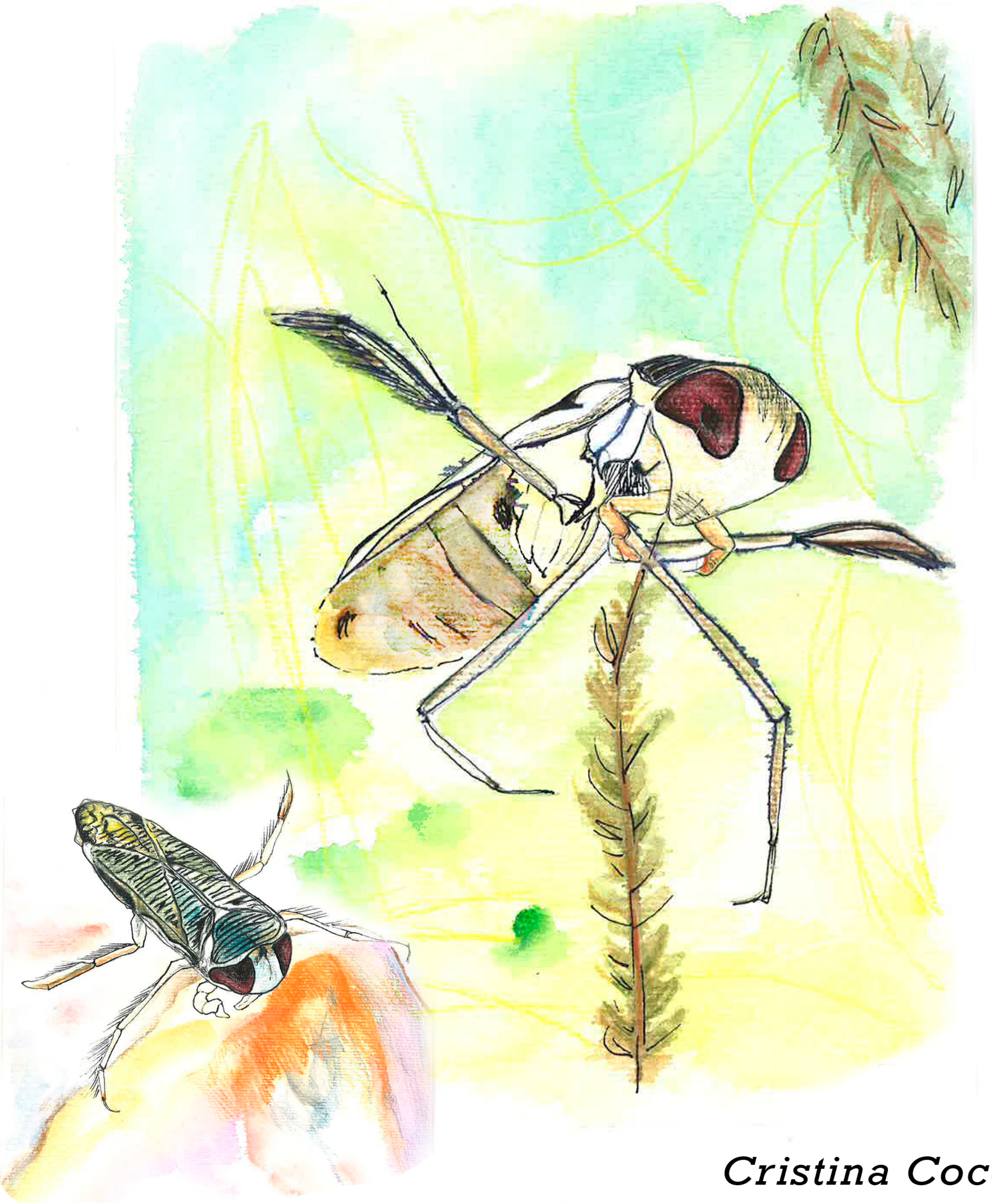
PhD thesis

Invasion biology of *Trichocorixa verticalis* in Doñana, SW Spain



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Invasion biology of *Trichocorixa verticalis* in Doñana, SW Spain

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Summary

Biological invasions are one of the top threats to biodiversity and ecosystem functioning worldwide, and fresh waters are among the most invaded ecosystems in the world. To be successful, an invader must possess qualities that allow invasion in the new habitat, but besides these qualities its success also depends on the interactions between its traits, the traits of the invaded community, and many other contingent factors. A recent addition to the list of alien invertebrate species in European fresh waters is the North American *Trichocorixa verticalis* (Hemiptera: Corixidae). To date, *T. verticalis* is the only established alien waterbug in these ecosystems. In the 18 years since its first detection in the Iberian Peninsula, *T. verticalis* has increased its area of distribution in and around Doñana, in the Guadalquivir delta, and also in other areas of conservation interest including Ramsar wetlands and Nature Reserves in Andalucia. In general, it is highly dominant and abundant in permanent saline waters, where native Corixidae are rare, but it is rare in fresh waters, where native Corixidae dominate.

This thesis focuses on four main determinants of successful invasions (plasticity, resource competition, parasites and facilitative interactions among invaders) to investigate why *T. verticalis* dominates in saline waters while it is rare in fresh waters. In addition, we explored the success of a restoration project for macroinvertebrates in new ponds where *T. verticalis* is known to be an abundant breeder. In addressing these topics, we apply an invasive-native comparative approach to both experimental and field data collected in Doñana.

In ***Chapter 1*** we show experimentally that *T. verticalis* possesses broader physiological plasticity than native corixids when exposed to different conditions of temperature and salinity, and its physiological tolerance to both heat and freezing increases following exposure to high conductivities. In ***Chapter 2*** we investigate the niche partitioning between native and invasive corixids from different ecosystems by means of carbon (C) and nitrogen (N) stable isotopes. We reveal strong resource partitioning between species in permanent ponds, but also some degree of niche overlap in unstable temporary sites. In ***Chapter 3*** we describe the role of parasite infections during the invasion. *T. verticalis* show higher total parasite (water mite) prevalence, mean total abundance infection and mean infection than native corixids in low salinity waters, whereas mites are not present in saline waters. In ***Chapter 4*** we examine experimentally the role of facilitative interactions among invaders. *T. verticalis* invasion does not seem to be promoted by a lower predation rate by alien predators compared to native corixids. In contrast, owing its smaller size it suffers higher predation rates by Odonata larvae. Finally, in ***Chapter 5*** we investigate the value of the Caracoles restoration project in supporting aquatic macroinvertebrates. *T. verticalis* is known to be an abundant breeder in these ponds. We find that, although new ponds differ from reference sites in abiotic conditions, they become representative and even surpass the levels of local invertebrate richness, diversity and abundance 6-7 years after restoration. However, differences in the abundance and distribution of invasive species between waterbody types and inundation periods may have strong effects on the patterns of species composition, especially for the Hemiptera.

The integration of these results sheds light on the role of salinity for the invasion success of *T. verticalis,* and helps to elucidate why it is still rare in fresh waters. In addition, we also provide important insights on the potential impacts that *T. verticalis* may have on native Corixids in the future.

Resumen

Globalmente, las invasiones biológicas constituyen una de las principales amenazas para la biodiversidad y el funcionamiento de los ecosistemas, y son los sistemas de agua continentales los mas susceptibles a estas invasiones. El éxito de una especie como invasora depende de sus cualidades, las que le permiten invadir un nuevo hábitat, pero también de las interacciones de éstas con la comunidad invadida, además de otros muchos factores. Recientemente, el coríxido de Norte América *Trichocorixa verticalis* (Hemiptera: Corixidae) ha sido incluido en la lista de invertebrados acuáticos invasores en los ecosistemas europeos. Hasta hoy, se trata de la única especie de heteróptero acuático invasor en estos ecosistemas. Tras 18 años desde su primera detección en la Península Ibérica, su área de distribución se ha expandido dentro y fuera de Doñana, encontrándose en el delta del Guadalquivir, así como en otros lugares de interés para la conservación en Andalucía, incluyendo humedales RAMSAR y Reservas Naturales. Por lo general, *T. verticalis* domina en aguas salinas donde los coríxidos autóctonos son poco comunes, pero es rara en aguas dulces donde los coríxidos autóctonos son dominantes.

La presente tesis investiga el papel de cuatro factores principales involucrados en una invasión (plasticidad, competencia trófica, parásitos e interacción entre especies exóticas) para comprender el éxito de *T. verticalis* en aguas salinas y su escasa presencia en aguas más dulces. Además, examinamos su potencial impacto en las comunidades de macroinvertebrados acuáticos en lucios artificiales que fueron creados durante el proyecto de restauración de humedales realizado en 2005 en Doñana, en los que sabemos que *T. verticalis* se reproduce abundantemente. Para abordar estos cuatro factores, usamos un método comparativo especie exótica frente a especies autóctonas aplicado a datos de campo y experimentales.

En el ***Capítulo 1***, detectamos experimentalmente, que *T. verticalis* tiene una mayor plasticidad fisiológica cuando es expuesta a diferentes condiciones de temperatura y salinidad, y que su tolerancia al calor y al frío aumenta después de ser aclimatada a elevadas salinidades. En el ***Capítulo 2***, investigamos mediante el uso de isótopos estables de Carbono (C) y Nitrógeno (N), la partición de recursos entre la especie exótica y las especies autóctonas de coríxidos, en diferentes ecosistemas. Encontramos una fuerte segregación en los recursos usados en lagunas permanentes, pero también un cierto nivel de solapamiento en los nichos isotópicos en lagunas temporales. El ***Capítulo 3***, describe el papel de las infecciones realizada por parásitos en esta especie invasora. En agua dulce, la prevalencia total, la abundancia total y la infección media de parásitos es mayor en *T. verticalis* que en los coríxidos

autóctonos. En aguas salina no hemos encontrado parásitos. En el ***Capítulo 4***, nos centramos en el papel de las "*facilitative interations*" entre especies exóticas. Descubrimos que *T. verticales* no sufre una menor tasa de depredación en comparación con los coríxidos autóctonos por parte de depredadores exóticos. Al contrario, debido a su menor tamaño, *T. verticalis* parece sufrir una mayor tasa de depredación por parte de larvas de Odonatos nativos. Por último, en el ***Capítulo 5***, investigamos el éxito de las comunidades de macroinvertebrados acuáticos en los ecosistemas restaurados de Caracoles, donde sabemos que *T. verticalis* cría abundantemente, por tanto su presencia podría influir en la recuperación de los macroinvertebrados acuáticos. Los resultados muestran que 6-7 años después de la restauración, los lucios artificiales, a pesar de tener condiciones abióticas muy diferentes en comparación con los sitios naturales de referencia, tienen el mismo nivel local de riqueza taxonómica y diversidad, y mayor abundancia que los sitios de referencia. Sin embargo, las diferencias en la abundancia y distribución de *T. verticalis* entre los lucios artificiales y los de referencia, a lo largo de los dos años de estudio, podrían explicar las diferencias anuales encontradas en la composición de especies de hemípteros. La combinación de estos resultados revelan el papel que el agua salobre tiene en el éxito de invasión de *T. verticalis* y aclaran el porqué de su rareza en agua dulce. Además, los resultado de esta tesis proporcionan información útil acerca del potencial impacto que *T. verticalis* podria tener sobre especies nativas de coríxidos en el futuro.

General Introduction & Outline

Biological invasion problem

Humans have moved other species from site to site for centuries (Wilson et al., 2009). However, their role in shaping the biota increased exponentially over time, especially throughout the 20th century (Hulme, Pysek & Nentwig, 2009). Many of these species, once introduced in habitats outside their native range (i.e., alien species), establish self-sustaining populations within the new environment (i.e., they become naturalized), but remain small and localized (i.e., non-invasive), while others spread widely and become abundant at many sites (i.e., they become invasive). Nowadays, only a few habitats are free of alien species, and in most of the systems they have invaded they now account for a significant portion of the biota (Vitousek et al., 1997).

Although not all alien species have appreciable effects on the invaded ecosystems, many of them have been often implicated in species extinction, habitat degradation and ecosystem alteration (Cox, 1999; Clavero & García-Berthou, 2005; Pimentel, Zuniga & Morrison, 2005). One classical example is the introduction of the piscivorous Nile perch (*Lates niloticus*) in the 1950s in Lake Victoria in East Africa, which led to the extinction of over 200 endemic fishes (Kitchell et al., 1997). There is a large consensus among researchers that biological invasions are one of the top threats to biodiversity and ecosystem functioning worldwide (Rahel & Olden, 2008). The increasing awareness to this global impact makes biological invasion a hot topic for research. To date, the extensive online information networks on invaders underpin the increasing necessity to improve information and impacts to be used in prevention and control actions (Simpson et al., 2006).

Freshwaters: one of the most invaded ecosystems in the world

Fresh waters are being subject to periodic deliberate and accidental introduction of alien species globally. Typical sources of invader introductions are ballast water, pet, aquarium and ornamental trade, sport fishing and research. In addition, exploitation and pollution of these waters and conversion to agriculture or urbanization also increase the likelihood of the alien species establishment and spread worldwide throughout degraded habitats (Rahel, 2002; MEA, 2005; Dudgeon et al., 2006). All together these factors convert fresh waters into one of the most invaded and threatened ecosystem in the world (Darwall et al., 2008; Strayer, 2010), with proportionally more invaders than terrestrial systems (Vitousek et al., 1997). Although insects form a large part of native fauna in freshwaters worldwide (Balian et al., 2008), the majority of invaders in these habitats belong to bivalves, gastropods and crustaceans (Cobo et al., 2010), while invasive aquatic insects are quite rare (Karatayev et al., 2009). Some possible explanations include the lack of their deliberate introductions as pet, ornamental trade etc., their inability to survive in active and diapausing stage in ballast waters or sediments, and their need to find suitable aquatic and terrestrial environments to complete their life cycle, often being aquatic as juveniles and terrestrial as adults (Duggan et al., 2005; Karatayev et al., 2009).

Among freshwater invaders, only a handful of them have recognized ecological impacts (e.g. the water hyacinth *Eichhornia crassipes*, the crayfish *Procambarus clarkii* and the zebra mussel *Dreissena polymorpha*, see Gherardi, 2006; Laranjeira & Nadais, 2008 and Higgins & Vander Zanden, 2010), while in general the consequences of alien species for faunal composition, community structure and ecosystem functioning in freshwater systems are largely unknown. Freshwater habitats hold more than 7% of described species (Darwall et al., 2008) with extensive local endemism (Gibon, 2000; Dudgeon, 2003). They are also vital for species that depend indirectly on them, including humans. Therefore, understanding the factors leading to successful invasion should be a priority of research, especially for taxonomic groups that include few invaders, such as aquatic insects, because this could provide predictive power for future invasions and important insights for the management of currently invaded systems.

Invasion process

Biological invasion is a multistage process that starts when individuals are transported outside their native area, become established and persisted in the new range (naturalized), proliferate and finally spread (invasion) (Kolar & Lodge, 2001). Several barriers characterize each one of these stages. If a species fails to pass one of these barriers, at any stage of the invasion process, it fails to become an invader. As a consequence, not all the introduced species become successful invaders, instead only a small percentage of transported taxa become established, and among these only about 1% become so abundant to dominate and even displace natives (Williamson & Fitter, 1996).

To be successful an invader must possess qualities that allow invasion in the new habitat, but besides these qualities its success is also the result of the interactions between its attributes, the attributes of the invaded community, and many other contingent factors (Lonsdale, 1999; Kolar & Lodge, 2001; Vilà & Weiner, 2004). Focusing on the processes that affect local establishment and spread in novel areas, invasive species usually arrive in sites with different environmental conditions than those in which they evolved; therefore they need to possess characteristics that first enable them to tolerate and then to adapt to the novel conditions. Species that have broad environmental tolerance (i.e., ecological breadth) should be able to sustain and maintain population growth over a wide range of conditions, a factor that may increase their chance to become invasive. The extent of such ecological breadth may be partly explained by their capacity to show plastic response to the environment (Sultan, 2001; Richards, Pennings, & Donovan, 2005). Plasticity, the *"environmentally sensitive production of alternative phenotypes by given genotypes"* (Hulme, 2008), has been indicated to play an important role during invasion (Richards et al., 2006; Ghalambor et al., 2007). Plastic responses can be induced by abiotic (e.g., light, temperature, nutrients) and biotic factors (e.g., predators, competitors), and can be expressed as behavioural, morphological and physiological changes (Reylea, 2001; Pigliucci, Murren & Schlichting, 2006).

Successful invasions also depend on the invaders' ability to exploit resources (Seabloom et al., 2003). From the time of release, invaders interact with species in the invaded systems. If invaders and natives are taxonomically similar and/or have similar diet, competition for limiting resources, such as space or food, is the more predictable consequence on the invaded community (Vilà & Weiner 2004; Dick, 2008). Invasive species are frequently considered superior competitors; examples include the zebra mussel *Dreissena polimorpha* outcompeting native bivalves, and the mosquitofish *Gambusia holbrooki* outcompeting the autochthonous Spanish toothcarps (Ricciardi, Neves & Rasmussen, 1998; Rincón, 2002).

The role of parasites during an invasion process has been widely recognized, but the mechanisms through which they can operate are different. For example, invaders can benefit from the scarcity of natural predators and pathogens, which are lost during the introduction process, compared to the native range – 'Enemy release hyphothesis' (Torchin, Lafferty & Kuris, 2002; Torchin et al., 2003; Keane & Crawley, 2002; Colautti et al., 2004; Prenter et al., 2004). By contrast, invaders may also introduce new parasites or pathogens that arrive with them, with detrimental effects for the native communities – 'Parasites Spillover' (Dobson & Foufopoulos, 2001; Power & Mitchell, 2004). Finally, invasive species can be suitable hosts for native parasites. In this case the impact can be either for the invasive host or for the native species, because invaders can also amplify the infection "spillback" from exotic to native species (Daszak, Cunningham & Hyatt, 2000; Tompkins & Poulin, 2006).

In multiply invaded ecosystems, such as fresh waters, new invaders have a great probability to arrive in sites already occupied by older invaders. In such a situation, the presence of the initial invader may facilitate the establishment of the new invader, especially if they share a common geographic or evolutionary history, as under the 'invasional meltdown hypothesis' (Simberloff & Holle, 1999). Facilitative interactions have been indicated as frequent as any other biotic interaction including competition, parasitism and predation (Bruno, Brombertg & Bertness, 2005), therefore they can play a key role during invasion success (Simberloff & Holle, 1999; Richardson et al., 2000).

Over the years, it has become increasingly clear that invasions are likely to result of multiple interacting mechanisms (Gurevitch et al., 2011). Multiple studies should therefore be integrated for a better understanding of the whole invasion process.

The case of *Trichocorixa verticalis*

In European fresh waters few insect species have been detected as invaders (Karatayev et al., 2009) and, among them, *Trichocorixa verticalis verticalis* (Fieber, 1851) (hereafter *T. verticalis*) is the only established alien waterbug (Rabitsch, 2008).

T. verticalis (Heteroptera, Corixidae) is a small aquatic insect (< 5.5 mm) naturally distributed along the Atlantic coast of North America from Labrador to the north of Mexico and some Caribbean islands (Sailer, 1948; Jansson, 2002). Recently it has been recorded in aquatic systems outside its native range in Africa, Oceania and Europe (Jansson & Reavell, 1999; Kment, 2006; L'Mohdi et al., 2010), although the geographic origin of these invasive populations is still not clear. It was supposed that the introduction of *T. verticalis,* particularly in SW Europe or South Africa and New Caledonia, was accidentally linked to the introduction of the fishes *Fundulus heteroclitus* or *Gambusia affinis,* respectively, which are sympatric with *T. verticalis* in its native area (Jansson, 1982; Jansson & Reavell, 1999; Sala & Boix, 2005). Recently the link between its actual distribution with the major maritime trade routes made plausible thinking that it was most likely dispersed by ship transport (Guareschi et al., 2013). *T. verticalis* is a euryhaline insect (Hutchinson, 1993) that is abundant, as adult and juvenile (Kelts, 1979), in brackish and saline waterbodies (Sailer; 1948). However, it is able to colonize several kinds of habitat, including rivers, brackish pools, potholes, and salt marshes (Günter & Christmas, 1959). In its native area *T. verticalis* overwinters as eggs, and hatching commences in spring and early summer (Tones, 1977; Kelts, 1979). After hatching, its life cycle includes six stages: five juvenile instars (each lasting 5–10 days) and adult (Fig. 1). It has two or three generations per year (Tones, 1977; Kelts, 1979). It is considered omnivorous and its diet is composed of both animal and plant sources, including zooplankton, filamentous algae and dipteran larvae (Kelts, 1979; Simonis, 2013). However, it seems a voracious predator of anostraceans and cladocerans (Wurtsbaugh, 1992; Simonis, 2012), and this may strongly influence ecosystem functioning. For example, field experiments showed that the consumption of cladocerans by adults of *T. verticalis* caused strong top-down trophic cascades (Simonis, 2013), and combined field and observational studies revealed that its predation on brine shrimp *Artemia franciscana* Kellog, 1906 during periods of low salinity affected the food web of the Great Salt Lake (USA) (Wurtsbaugh 1992).

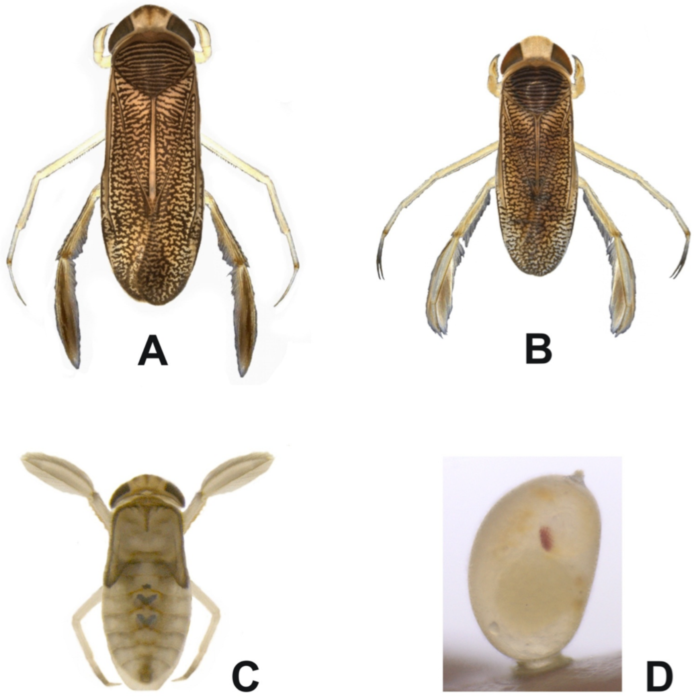

Figure 1. *Trichocorixa verticalis* female (A), male (B), V instars nymph (C) and egg (D) (from Guareschi, PhD Thesis, 2015).

Introduction in the Iberian Peninsula

In the Iberian Peninsula, *T. verticalis* can clearly be considered invasive. It was first recorded in Algarve (Portugal) in 1997, but soon thereafter it was found at several sites along the Iberian Atlantic coast (Sala & Boix, 2005), in various Andalucian wetlands, including Ramsar sites (Millán et al., 2005; Rodríguez-Pérez et al., 2009; Van de Meutter, Trekels & Green, 2010a, authors unpublished data, see Fig. 2), and bio-climatic models have predicted its future spread across Europe and the Mediterranean region (Guareschi et al., 2013). In and around Doñana in the Guadalquivir delta *T. verticalis* is highly dominant and abundant in permanent saline fish ponds (especially the Veta la Palma estate) and salt ponds, where native Corixidae are rare, in contrast to lower salinity waters, where the native corixids dominate but *T. verticalis* is rare (Rodríguez-Pérez et al., 2009; Van De Meutter, et al., 2010). *T. verticalis* is also highly dominant in the salt ponds within the Odiel marshes and Cádiz Bay, as well as in coastal ponds along the Algarve.

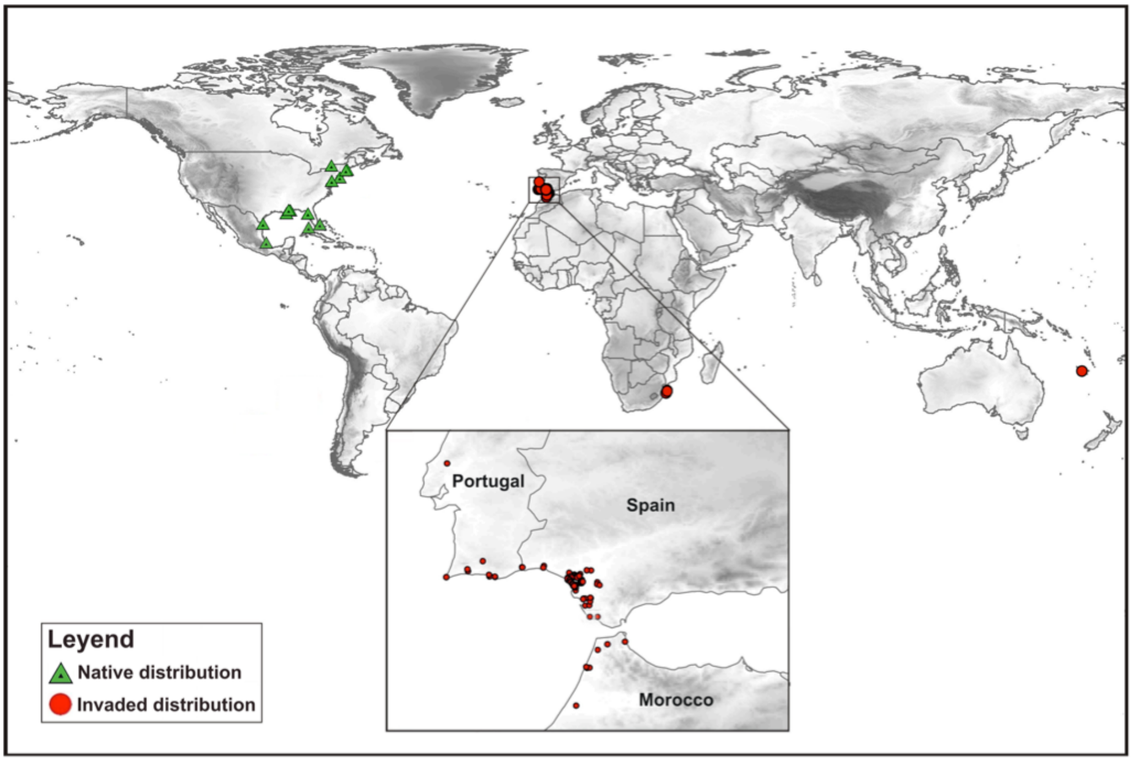

Figure 2. Map representing the current distribution of *Trichocorixa verticalis* in the native (triangles) and invaded (circles) areas (from Guareschi et al., 2013).

The ability of *T. verticalis* to live in hypersaline waters, as juvenile and adult, has been suggested to play a key role in explaining its invasion success (Van De Meutter et al., 2010), allowing the fill of an empty niche. However, when compared through an experimental approach the salinity tolerance of adult *T. verticalis* did not differ from the tolerance of native corixids, especially respect to *Sigara selecta* (Van De Meutter, et al., 2010). This suggests that the successful invasion of *T. verticalis* can not be simply explained on the basis of osmoregulation ability, and that other factors such as e.g. release from enemies, higher plasticity and/or higher competitive ability than native corixids may account for its success.
To date, there are few data on the potential ecological impact of *T. verticalis* on native aquatic communities and ecosystems in its introduced range. In the Larache salt ponds in Morocco, L'Mohldi and co-workers (2010) observed that increased abundance of *T. verticalis* coincided with a decrease in the abundance of the native *S. selecta*. This suggests that in the Iberian Peninsula, where *T. verticalis* dominates and native Corixidae are rare, the latter may have been competitively excluded. This seems especially true for *S. selecta* that share similar habitat requirements with *T. verticalis.* However, *T. verticalis* may also affect, directly or

indirectly, the native Corixidae often found with them – *Sigara lateralis*, *S. stagnalis*, and *S. scripta* (Rodriguez-Perez et al., 2009) –given its ability to persist in a wide range of habitats, its high voracity combined with its generalised feeding habitats.

Specifically, in this thesis we will focus mainly on the comparison between *T. verticalis* and the *Sigara lateralis* (Leach, 1817). *S. lateralis* (Fig. 3) is a Paleartic, opportunistic species frequently found inhabiting in small temporary freshwater ponds (Millán et al., 1988; Boda & Csabai, 2009; (Cianferoni, 2009). It is a corixid of 5-6 mm length (Nieser, Baena, Martínez-Avilés & Millán, 1994) with at least two generations per year (i.e. bivoltine, Cianferroni, 2009). This species is known to feed mainly on animal prey (Murillo & Recasens, 1986; Layer et al., 2010) and to possess high dispersal ability (Boda & Csabai, 2009).

Figure 3. *Sigara lateralis*
(Photo: www.biodiversidadvirtual.org).

Invasive species can bring serious threats to the conservation of protected areas (Lonsdale, 1999; Lovejoy, 2006). The cordgrass *Spartina densiflora,* the crayfish *Procambarus clarkii* and the water hyacinth Eichhornia crassipes are three good examples of invaders that had detrimental effects on protected ecosystems (Castillo et al., 2008; Cruz et al., 2008; Laranjeira & Nadais, 2008). Doñana marshes have a critical role in biodiversity conservation and represent one of the most important breeding and overwintering sites for many migratory water birds. The successful establishment of *T. verticalis* in this area could represent the latest menace added to these aquatic systems. Like many other wetlands in the Mediterranean region, the Doñana marshes have been subject to strong anthropogenic impacts since 1920, including increases in water demand that followed agriculture, tourism and urbanization development outside Doñana borders (Martín-López et al., 2011), changes in the inundation regime over the years and also multiple invasions (Frisch, Rodríguez-Pérez & Green, 2006; Garcia-Murillo et al., 2007; Rodríguez-Pérez et al., 2009; Bravo Utrera, 2010). For most of these invaders, the effects on wetland ecosystem functioning and on the conservation of native fauna have been investigated in some detail, while for others, especially for the aquatic insects *T. verticalis,* these are largely unknown. Studies on insects would be particularly valuable because they often cause substantial ecological change and economic damages (Kolar & Lodge, 2001).

In some cases, invasive species are also the cause of failure in restoration projects. For example, Matthews & Spyreas (2010) showed that when the abundance of the invasive reed canary grass *Phalaris arundinacea* increased, the vegetation communities within restored wetlands progressed towards an undesirable community state. *T. verticalis* has a well-established population within the "Caracoles estate" in Doñana National Park (South West Spain), a large complex of new ponds that were created during marsh restoration (Rodriguez-Perez et al., 2009; Frisch et al., 2012). Since *T. verticalis* could affect native species directly via competition and predation, or indirectly by modifying the environmental conditions, this species may therefore interfere with a successful recovery of aquatic macroinvertebrate diversity in these new ponds, when compared with natural communities.

Objectives and thesis outline

What determines invasiveness of alien organisms and which are the potential consequences of a biological invasion on the host community are among the most interesting and urgent questions in ecology (Levine et al., 2002, 2003; van Kleunen et al., 2010). In this thesis we used an invasive-native comparative approach on both experimental and field data collected in Doñana. We explore the hypothesis that the invader *T. verticalis* dominates in saline waters, but is rare in fresh waters, for various reasons: (1) it is more plastic than native species and its physiological performance improves at high salinity; (2) it is released from natural enemies (native parasites, predators); (3) its spread is facilitated by interactions with alien fish predators; and (4) its trophic relationships with native corixids differ between saline and fresh waters. Lastly, we explored the success of a restoration project for macroinvertebrates in new ponds where *T. verticalis* is known to be an abundant breeder.

Since plasticity is generally a characteristic of good invaders, in ***Chapter 1*** we experimentally compare thermal tolerance and plasticity responses between *T. verticalis* and the native *S. lateralis* acclimated to different conditions of salinity and temperature. In ***Chapter 2*** we combine experimental and field data on stable isotopes of carbon (C) and nitrogen (N) to investigate the potential of *T. verticalis* to compete for food with native corixids in habitats that differ in water stability and resource diversity. We also investigate the trophic ecology of this invader where it has stable reproductive populations within Doñana. To evaluate the role of parasites during this invasion, in ***Chapter 3*** we compare larval water mite infection levels between native corixids and *T. verticalis* along a salinity gradient combining specific and generic parasite sampling. As facilitative interactions among exotics have been shown to play an important role during invasion, in ***Chapter 4*** we experimentally investigate differences in predation rates between two exotic predators and one native predator on the invasive and native corixids. In ***Chapter 5*** we evaluate the restoration success of the Caracoles ponds, where the invasive species has reproductive populations, for macroinvertebrates (the whole community, Coleoptera and Hemiptera) compared to natural reference sites during two different inundations, so as to depict the potential impact of *T. verticalis* within a restoration framework. Finally, we synthesize and discuss the major results of the previous chapters and we report the conclusions of this thesis.

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Chapter 1

Does ecophysiology determine invasion success? A comparison between the invasive boatman *Trichocorixa verticalis verticalis* and the native *Sigara lateralis* (Hemiptera, Corixidae) in south-west Spain

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Abstract

Trichocorixa verticalis verticalis, a native of North America, is the only alien corixid identified in Europe. First detected in 1997 in southern Portugal, it has spread into south-west Spain including Doñana National Park. Its impact on native taxa in the same area is unclear, but it is the dominant species in several permanent, saline wetlands.

We investigated whether the ecophysiology of this alien species favours its spread in the Iberian Peninsula and its relative success in saline areas. We compared physiological responses to heating (Critical Thermal maximum), cooling (Critical Thermal minimum) and freezing (Super Cooling Point) in the native *Sigara lateralis* and introduced *T. verticalis* acclimated to different temperatures and salinities. The larger *S. lateralis* generally outperformed *T. verticalis* and appeared to possess a broader thermal tolerance range. In both taxa, CT_{max} was highest in animals exposed to a combination of high conductivities and relatively low acclimation temperatures. However, CT_{max} was generally higher in *T. verticalis* and lower in *S. lateralis* when acclimated at higher temperatures. CT_{min} were lower (greater tolerance to cold) after acclimation to high conductivities in *T. verticalis*, and following acclimation to low conductivities in *S. lateralis*. Both acclimation temperature and conductivity influenced corixids´ freezing tolerance; however, only in *T. verticalis* did SCP decrease after exposure to both high temperature and conductivity. *T. verticalis* showed a higher range of mean responses over all treatments.

Whilst the native *S. lateralis* may have a broader thermal range, the alien species performs particularly well at higher salinities and temperatures and this ability may facilitate its invasion in Mediterranean areas. The greater plasticity of *T. verticalis* may further facilitate its spread in the future, as it may be more able to respond to climate shifts than the native species.

Introduction

Freshwater habitats occupy less than 1 % of the world's surface, but hold more than 7 % of described species (Darwall et al., 2008), with extensive local endemism (Gibon, 2000; Dudgeon, 2003). At the same time, however, inland water ecosystems and biological communities are affected by increasing numbers of alien species (Cohen, 2002) and are amongst the most threatened in the world (Darwall et al., 2008). According to the DAISIE database, there are 296 invertebrate alien species in European inland waters (Gherardi et al., 2009). However, the consequences of invasive invertebrate species for faunal composition, community structure and ecosystem functioning in freshwater systems are largely unknown, with the exception of a handful of taxa such as the red swamp crayfish *Procambarus clarkii* (Gherardi, 2006) and the zebra mussel *Dreissena polymorpha* (Higgins & Vander Zanden, 2010).

Whilst some taxonomic groups (e.g. bivalves, crustaceans and gastropods) are well represented in alien invertebrate species lists, insects are highly under-represented, despite them dominating the world's freshwater fauna (Balian et al., 2008). A recent addition to these lists is the water boatman *Trichocorixa verticalis verticalis* (Fieber, 1851) (Heteroptera, Corixidae), native to North America, but now occurring in temperate zones in other parts of the world such as South Africa, Iberia and Morocco (Kment, 2006; Jansson & Reavell, 1999; L'Mohdi et al., 2010). In Europe, *T. verticalis* represents the only established alien waterbug (Rabitsch, 2008). In the Iberian Peninsula it was first recorded in 1997 in the Algarve in Portugal (Sala & Boix, 2005). It is now successfully established and continues to spread, but is so far restricted to areas along the Atlantic coast (Sala & Boix, 2005) and in the Guadalquivir Estuary and surrounding parts of SW Spain (Rodríguez-Pérez et al., 2009; Van De Meutter et al., 2010). It is predicted to spread widely across Europe and the Mediterranean region in the future (Guareschi et al., 2013).

T. verticalis is now the dominant breeding corixid at several sites in and around Doñana National Park on the Guadalquivir Estuary (Rodríguez-Pérez et al., 2009; Van De Meutter et al., 2010). Part of its success appears to be related to its ability to live in hypersaline environments (Kelts, 1979), and to colonize different kinds of habitats, including brackish and saline waterbodies (Günter & Christmas, 1959). This ability may enhance the competitive advantage of *T. verticalis* over other corixids in the face of global change. During the twentieth century, the wetlands in southern Spain and the rest of the Mediterranean region have become increasingly prone to development and extraction of fresh water (Green et al., 2002; Sousa et al., 2010) and these factors, together with projected climate-induced changes in hydrology, increase salt concentrations in remaining waterbodies (Moss et al., 2009).

If native species are unable to respond to extreme conditions, either physiologically (Calosi, Bilton & Spicer, 2008; Calosi et al., 2008; Calosi et al., 2010; Bozinovic, Calosi & Spicer, 2011) or behaviourally (Kearney, Shine & Porter, 2009; Tewksbury, Huey & Deutsch, 2008), they are likely to be excluded through interspecific competition with more tolerant species (Dick & Platvoet, 1996; Cáceres, 1998). Field data on the distribution of *T. verticalis* suggest that its physiological tolerance of salinity may be at least partly responsible for its competitive advantage over native corixids in the Doñana area (Van De Meutter et al., 2010).

Moreover, the effects of salinity and temperature on insect physiological tolerance can be synergistic or additive. Sánchez-Fernández et al. (2010) for example, recently demonstrated how the interaction of these two environmental factors influences the thermal biology of adult *Nebrioporus* diving beetles, where cold tolerance increases following exposure to high salinities and low temperatures.

In this experimental study, we subjected *T. verticalis* and the native Palaearctic corixid *Sigara lateralis* (Leach, 1817) (Cianferoni, 2009) to different combinations of temperature and salinity and compared several indicators of upper and lower thermal sensitivity of individuals of both species acclimated to different conditions. These two species are sympatric in southern Iberia, and frequently occur together in the same ponds, although *T. verticalis* is becoming the dominant corixid in some areas previously occupied by *S. lateralis* (Rodríguez-Pérez et al., 2009). We specifically examined their critical thermal maximum (as a proxy for upper thermal limits), chill coma (as a proxy for lower thermal limits) (Huey et al., 1992; Castañeda, Lardies, & Bozinovic, 2005), and cold hardiness (supercooling point, often used as a measure of tolerance to low temperatures) (Sinclair & Sjursen, 2001; Worland & Convey, 2001). Differences in thermal tolerance and plasticity between native and invasive species can be used as predictors of their ability to persist, increase or decline in response to climate change. We explore whether exposure to different acclimation salinities and temperatures influence the thermal tolerance of the native and invasive species in an interactive manner, and examine the implications these have for the spread of *T. verticalis*.

Materials & Methods

Animal collection and maintenance

Adults of *T. verticalis* and *S. lateralis* were collected during July and August 2010 using a D-framed pond net (500 μm mesh; 16 × 16 cm) from different sites in Doñana and the Odiel marshes (SW Spain). Permits for sampling in Doñana and Odiel were provided by the

Consejería de Medio Ambiente, Junta de Andalucía. Conductivity of sampling sites ranged from 60 mS cm^{-1} (Odiel marshes) to 1.15 mS cm^{-1} (Doñana National Park) (See Table 1). Sites were chosen based on preliminary observations of corixid presence (Rodríguez-Pérez et al., 2009), authors' unpublished data). After collection, corixids were transported to the laboratory inside plastic containers filled with damp aquatic vegetation and kept within thermally insulated polystyrene boxes in order to minimize thermal fluctuations and extremes as much as possible. In the laboratory, individuals were maintained in aquaria containing water close to the original conductivity, before being transferred to holding aquaria with water at conductivity 18 mS cm^{-1}. When the original conductivity was >35 mS cm^{-1}, to avoid acute exposure to experimental conditions, individuals were first maintained at 25-30 mS cm^{-1}, before being transferred to 18 mS cm^{-1} (see Table 1). Aquaria were provided with sand and vegetation, and corixids were fed *ad libitum* with frozen chironomid larvae. Individuals were maintained on a natural photoperiod regime for 24 h before they were subjected to acclimation conditions, with a 12 h: 12 h D:L regime.

Table 1. Collection sites in SW Spain, original conductivities and maintenance water conditions in the laboratory**.**

Sampling date	**Arrival date**	**Sites**	**Original conductivity**	**Laboratory conductivity**	**Species**
21/07/10	23/07/10	Veta la Palma (VLP)	11.59 mS cm^{-1}	12 mS cm^{-1}	*Tv* + *Sl*
29/07/10	02/08/10	Odiel Marshes	60 mS cm^{-1}	30 mS cm^{-1}	*Tv*
30/07/10	02/08/10	VLP-EBD	14 mS cm^{-1}	14 mS cm^{-1}	*Tv*
30/07/10	02/08/10	FAO	2.8 mS cm^{-1}	2.6 mS cm^{-1}	*Tv* + *Sl*
30/07/10	02/08/10	Caracoles	41 mS cm^{-1}	25 mS cm^{-1}	*Tv* + *Sl*
19/08/10	20/08/10	FAO	1153 µS cm^{-1}	1 mS cm^{-1}	*Tv* + *Sl*
19/08/10	20/08/10	VLP-EBD	36.5 mS cm^{-}	25 mS cm^{-1}	*Tv*
31/08/10	01/09/10	FAO	1.32 mS cm^{-1}	1.32 mS cm^{-1}	*Tv* + *Sl*
31/08/10	01/09/10	Caño Guadiamar	6.76 mS cm^{-1}	6 mS cm^{-1}	*Tv* + *Sl*

All sites are in Doñana except the Odiel Marshes (See Rodríguez-Pérez et al., 2009; Van De Meutter et al., 2010 for details). VLP-EBD are individuals reared in mesocosms at the EBD (Estacíon Biólogica de Doñana-CSIC) but originating from Veta la Palma. *Tv= Trichocorixa verticalis*; *Sl= Sigara lateralis.*

Experimental setup and acclimation

Individuals were transferred to 3 L aquaria (with a maximum of 13 ind. of the same species in each aquarium) at 4 different conductivities: 1, 4, 12 and 18 mS cm^{-1}, which corresponded to salinities of 0, 2.1, 6.8 and 10.6 ppt. Aquaria were kept for 72 h in either a climatic chamber set at 10 or 15 ºC or a water bath set at 25 °C. Temperatures and salinities were chosen to simulate a range of conditions present at waterbodies where both species are found together (Rodríguez-Pérez et al., 2009; Van de Meutter, Trekels & Green, 2010). Whilst these conductivities do not span the entire range occupied by *T. verticalis* in the field (see above) they were chosen since preliminary experiments demonstrated that they were non-lethal in both taxa studied, allowing direct comparison of their responses to be conducted across a wide conductivity range. Waters of different conductivity were prepared by dissolving an appropriate quantity of salt (Instant Ocean, Aquarium Systems, Sarrebourg, France) in aerated artificial pond water, that consists of a solution of salts dissolved in double-distilled water, prepared according to a standardized protocol (ASTM, 1980). During the experiment we monitored water temperature and conductivity at 12 h intervals using a handheld multimeter (YSI 85, Yellow Springs, USA). Conductivity fluctuations, due to evaporation and/or differences in solubility, were corrected by dissolving small quantities of Instant Ocean or adding artificial pond water to aquaria. Aquaria were sealed with cling-film to reduce evaporation and to prevent individuals from escaping, whilst aeration was continuously provided. No food was provided 24 h prior to thermal tolerance limits being determined.

Following the exposure period, 10 individuals of each species were randomly removed from each treatment and further sub-divided into two equal-sized groups: one sub-group was used to measure critical thermal maximum (CT_{max}) and the other to measure critical thermal minimum (CT_{min}). The estimation of supercooling point (SCP) was undertaken in separate trials approx. 15 d after the determination of thermal limits, using the same procedure. After experiments, individuals were sexed using a stereo microscope and weighed to the nearest 0.001 g using a Sartorius 1204 MP2 balance (Sartorius Ltd, U.K.).

Thermal tolerance and supercooling point experiments were carried out in air given the impossibility to estimate freeze tolerance in water. This procedure provides an indication of the ability of a species to perform better than others at high or low temperatures in water as well as air (Calosi, Bilton & Spicer, 2008; Calosi et al., 2008; Calosi et al., 2010; Sánchez-Fernández et al., 2010).

Thermal tolerance experiments

Thermal tolerance tests commenced at the temperature at which individuals had been acclimated (see Terblanche et al., 2007 for methodological details). A total of 240 individuals were used: 120 *S. lateralis* and 120 *T. verticalis.* Individuals were removed from their acclimation aquaria, quickly but carefully blotted on absorbent paper, and placed into a clean and dry well of a plastic multiwell culture plate. For CT_{min}, specimens were placed individually into a generic 24-well plastic culture plate (Corning Ltd, Sunderland, UK), while for CT_{max} a modified plate was used with deeper wells to avoid escape during heating. In both cases, external bases were painted with white Tipp-Ex to allow easy visualization of temperature related responses. Plates were immersed in the water bath until only the upper edges (1- 2 mm) were exposed, and affixed to the side of the bath with adhesive tape to prevent movements and thus water entering experimental wells. To further avoid escape, well plates were covered with a plastic lid between additions of individuals. Once the experiment started, lids were removed to allow full aeration and avoid the build-up of water vapour, which might have affected the thermal tolerance of individuals (Pörtner, 2001). A maximum of 5 individuals were tested at any one time.

Thermal tolerance tests relied on a dynamic method, which involves increasing or decreasing test temperatures via a ramping program (± 1 °C min) until the end-point (see below) was observed. A rapid ramping rate was favoured as it allows observed responses to be related to the effect of different acclimations, and minimizes other undesired effects that may occur during slower ramping on thermal limits (see Rezende, Tejedo, & Santos, 2011). Experiments were performed with a Grant R5 water bath (12 l capacity) and a GP200 thermostatic controller (Grant Instruments Ltd., Cambridgeshire, England) connected to a computer. Grant Labwise software was used to construct and control temperature programs. The actual temperature within each well was measured directly using a calibrated digital thermometer (Omega_ HH11; Omega Engineering Inc., Stamford, CT, USA) equipped with an Omega® 'precision fine wire thermocouple' (type T – dia./ga. 0.08/0.13 Teflon). Distilled water and 70% ethylene glycol solutions were used as fluids inside the water bath to determine CT_{max} and CT_{min} /SCP respectively.

CT_{max} and CT_{min} were defined using individual end-points represented by death (lethal point) at high temperatures, and chill coma (sub-lethal point) at low temperatures. Whereas death was readily identifiable in CT_{max} experiments (individuals never revived after cessation of movement), defining lower lethal limits was more difficult. At low temperatures, individuals exhibited total paralysis and were apparently dead (chill coma), but they would revive and recover full or partial locomotory abilities shortly after the end of the exposure period. As already documented for other insects (Gaston & Chown, 2008; Calosi et al., 2010), both lethal limits and sublethal end-points (e.g. paralysis) provide an accurate picture of insect thermal biology. Consequently, we defined CT_{min} as the temperature at which individuals were paralysed, as the few corixids which recovered from the treatment were severely impaired in their locomotory ability and died shortly afterwards.

Supercooling point experiment

The SCP is the temperature of spontaneous freezing at which a biological solution or a whole organism freezes when cooled below its equilibrium freezing temperature (Salt, 1961; Wilson, 2003). During this experiment, the temperature at which individuals froze (SCP) was determined with a Campbell Scientific CR1000 datalogger equipped with an Omega 'precision fine wire thermocouple' (type T 1 mm long, 0.08 or 0.13 mm diameter) interfaced to a computer. Data were recorded and stored at 1 s intervals using Campbell Scientific PC400 software. Tests were carried out using a Grant R5 water bath (12 l capacity) and a GP200 thermostatic controller (Grant Instruments Ltd., Cambridgeshire, England) connected to a computer. Grant Labwise software was used to construct and control temperature programs.

A total of 115 individuals were tested: 60 *S. lateralis* and 55 *T. verticalis*. Individuals were removed from their exposure aquaria, quickly but carefully blotted on absorbent paper, and attached individually by the dorsum to an acetate disk with cyanoacrylic glue (Loctite, Henkel Ltd, Hempstead, UK). Individuals were introduced, one per well, into a 12-well plastic culture plate. A maximum of 5 animals were run concurrently in each experiment. The SCP was measured by supporting the thermocouple vertically on the insect's abdomen. Thermocouple movement was avoided by fixing individuals to the cell walls with BlueTack. Once ready, the individuals were transferred to the tank, and plates were covered with acetate lids to avoid thermal oscillations during the experiment. Individuals were cooled with a cooling ramp program (±1 °C min^{-1}), starting from the temperature at which individuals had been acclimated. The SCP of each individual was recorded as the lowest temperature reached before the start of the exothermic reaction caused by the latent heat of freezing of the animal's body fluids (Aarset & Torres, 1989; Worland, Leinaas & Chown, 2006). Owing to a shortage of individuals, we were unable to test the SCP on individuals of *T. verticalis* exposed to 25 °C and 18 mS cm^{-1}.

Statistical analyses

In order to assess the effect of exposure to different temperatures and conductivities on the thermal biology of *S. lateralis* and *T. verticalis*, we examined differences in CT_{max}, CT_{min} and SCP with general linear models on untransformed data; with acclimation temperature (10, 15 or 25 °C), acclimation conductivity (1, 4, 12 and 18 mS cm^{-1}), and species (*T. verticalis* or *S. lateralis*) as fixed factors, and sex (male or female) as a random factor. With the exception of CT_{max}, sex did not have a significant effect and was excluded from further analyses. Variances met assumptions for homoscedasticity (Levene's test, $P > 0.05$), and data met the assumption of normality (Shapiro–Wilks test, $P > 0.05$) for both CT_{min} and SCP as untransformed data, but not for CT_{max}, even after $\log_{10}$ transformation. However, given our sample sizes, models employed were robust to deviations from normality (Sokal & Rohlf, 1995; Underwood, 1997) and examination of residual plots for all data revealed satisfactory patterns. Model selection started by incorporating all predictors and the interactions between factors. Then, non-significant interactions were removed in a hierarchical, stepwise manner until a significant effect or interaction was found.

Body weight was not included in the overall model because it was not measured in all individuals of *T. verticalis*. We thus used a second model for only S. *lateralis* with the above factors together with body weight as a covariate. With the exception of CT_{max}, body weight did not have a significant effect on *S. lateralis* thermal limits ($P > 0.05$ for both CT_{min} and SCP), and was thus excluded from further analyses.

Finally, Bonferroni-corrected Estimate Marginal Means post-hoc tests were used for pairwise comparisons when any single factor or interaction was significant. All analyses were performed using SPSS version 17.0.

Results

Critical thermal maximum

For both species, mean CT_{max} reached its maximum when individuals were acclimated at the lowest temperature (10 °C) and the highest conductivity (18 mS cm^{-1}) (Figure 1), whilst minimum CT_{max} were recorded at 10 °C and 1 mS cm^{-1} for *T. verticalis* and 25 °C and 18 mS cm^{-1} for *S. lateralis.*

In terms of their CT_{max}, *S. lateralis* and *T. verticalis* responded differently to acclimation at different temperatures (temperature x species interaction $P < 0.001$; Figure 1, Table 2 - Bonferroni tests maximum $P = 0.035$; Supplementary Table 1). Mean CT_{max} was also significantly influenced by the interaction between temperature and conductivity ($P < 0.001$;

Supplementary Fig. 1, Table 2 - Bonferroni tests maximum *P* = 0.049; Supplementary Table 1). Sex also had a strong influence (*P* < 0.03; Table 2) on CT$_{max}$ in both species' heat tolerance, CT$_{max}$ being higher on average in females than males (Bonferroni tests maximum *P* = 0.030; Supplementary Table 1).

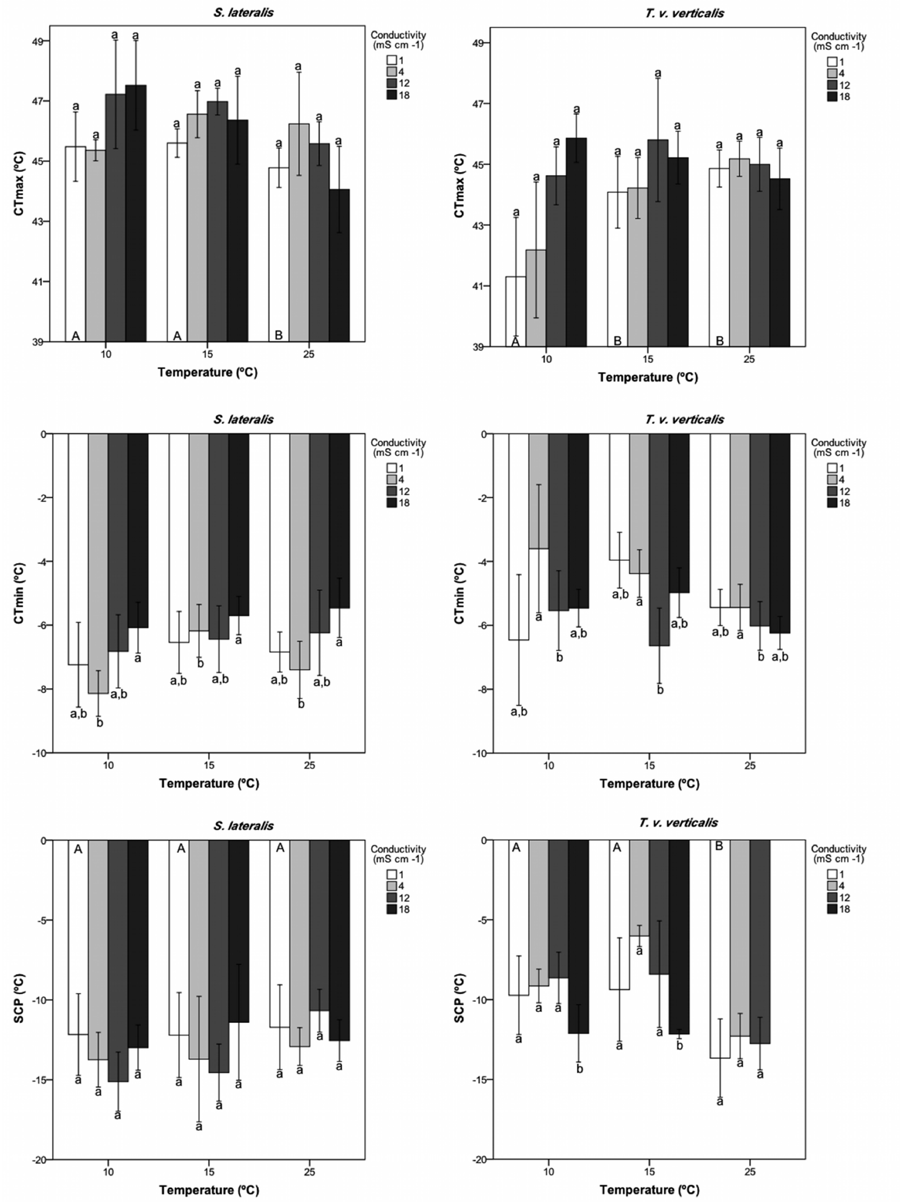

Figure 1. Thermal limits and freezing point of *Trichocorixa verticalis* and *Sigara lateralis***.** Histograms of mean ± SE critical thermal maximum (CT$_{max}$), critical thermal minimum (CT$_{min}$) and supercooling points (SCP) of *Sigara lateralis* and *Trichocorixa verticalis* acclimated to different temperatures (10, 15 and 25 °C) and conductivities (1, 4, 12, 18 mS cm^{-1}). Significantly different means within species (*P* < 0.05) measured at different acclimation temperatures are indicated by different capital letters inside the histograms, whereas significantly different means measured at different conductivities within the same temperature treatment are indicated by different lower case letters above or below the histograms (according to Estimated Marginal Means tests with Bonferroni correction).

Table 2. Effect of acclimation temperature (T), acclimation conductivity (C), species (Sp: *Trichocorixa verticalis* or *Sigara lateralis*) and sex on corixid critical thermal maximum (CT_{max}) – General linear model.

Source	**SS**	**d.f**	**MS**	***F***	***P***
T	10.3	2	5.1	2.92	0.05
C	37.2	3	12.4	7.03	< 0.001
Sp	51.8	1	51.8	29.37	< 0.001
Sex	8.5	1	8.5	4.85	0.03
T × C	59.2	6	9.8	5.60	< 0.001
T × Sp	32.7	2	16.3	9.28	< 0.001
C × Sp	3.7	3	1.2	0.71	0.54

Sum of squares (SS); degrees of freedom (d.f.); mean square (MS), F-ratio (*F*), probability level (*P*).

Overall, CT_{max} was significantly higher in *S. lateralis* than in *T. verticalis* (Supplementary Fig. 2) at 10 °C and 15 °C ($P < 0.05$) but not at 25 °C ($P > 0.05$). However, post-hoc comparisons showed that conductivity had a marginal influence on CT_{max} when animals were acclimated at higher temperatures. In contrast, CT_{max} was significantly lower for *S. lateralis* at 25 °C than at other temperatures (Fig. 1; Supplementary Table 1).

S. lateralis was larger on average than *T. verticalis*, with mean (± SD) body weights of 5.35 ± 1.28 mg and 3.46 ± 0.73 mg, respectively. When *S. lateralis* was analysed separately with body weight as an additional covariate (Table 3), CT_{max} increased significantly with body weight (Pearson correlation $R = 0.537$, $P < 0.001$) but sex no longer had a significant effect. Hence the effect of sex on CT_{max} seems to be a direct consequence of the lower body weight of males. Conductivity was the only other variable retaining a significant partial effect on CT_{max} once body weight was controlled for.

Table 3. Effects of acclimation temperature (T), acclimation conductivity (C) and weight (W) on the critical thermal maximum (CTmax) of *Sigara lateralis*– General linear model.

Source	**SS**	**d.f**	**MS**	***F***	***P***
T	6.4	2	3.2	2.43	0.098
C	17.1	3	5.7	4.34	0.009
W	18.2	1	18.2	13.88	0.001
T × C	11.7	6	1.9	1.40	0.206

Sum of squares (SS); degrees of freedom (d.f.); mean square (MS), F-ratio (*F*), probability level (*P*).

Critical thermal minimum

Minimum CT_{min} were recorded at 10 °C and 4 mS cm^{-1} for *S. lateralis* and 15 ºC and 12 mS cm^{-1} for *T. verticalis* (Fig. 1). Maximum CT_{min} were recorded at 25 ºC and 18 mS cm^{-1} for *S. lateralis* and at 10 ºC and 4 mS cm^{-1} for *T. verticalis*. Mean lower thermal limit was significantly influenced by the interaction between species and conductivity ($P < 0.001$; Table 4 - Bonferroni tests maximum $P = 0.006$; Table S2). Mean CT_{min} also differed significantly between species ($P < 0.001$) with *S. lateralis* showing a higher tolerance to cold than *T. verticalis* (Bonferroni tests maximum $P < 0.001$; Supplementary Fig. 2; Supplementary Table 2. Acclimation temperature was not significantly related to mean CT_{min} in either species.

CT_{min} was lower for *S. lateralis* (i.e., this species showed a greater tolerance to low temperatures) at both 1 and 4 mS cm^{-1}. For *S. lateralis*, CT_{min} increased significantly as conductivity increased from 4 to 18 mS cm^{-1}. In contrast, CT_{min} for *T. verticalis* decreased significantly as conductivity increased from 4 to 12 mS cm^{-1} (Supplementary Table 2).

Table 4. Effect of acclimation temperature (T), acclimation conductivity (C) and species (Sp: *Trichocorixa verticalis* or *Sigara lateralis*) on corixid critical thermal minimum (CTmin) – General linear model.

Source	**SS**	**d.f**	**MS**	***F***	***P***
T	8.0	2	4.0	2.7	0.067
C	6.7	3	2.2	1.5	0.208
Sp	46.3	1	46.3	32.	< 0.001
T × C	13.8	6	2.3	1.5	0.158
T × Sp	6.1	2	3.0	2.1	0.126
C × Sp	31.5	3	10.5	7.2	< 0.001

Sum of squares (SS); degrees of freedom (d.f.); mean square (MS), F-ratio (*F*), probability level (*P*).

Supercooling point

The minimum SCP for *S. lateralis* occurred when acclimated at 10 °C and 12 mS cm^{-1}, whilst the maximum for this species occurred when acclimated at 25 °C and 12 mS cm^{-1} (Fig. 1). For *T. verticalis*, minimum and maximum SCP occurred when acclimated at 25 °C and 1 mS cm^{-1} and 15 °C and 4 mS cm^{-1}, respectively (Fig. 1). Mean SCPs for *S. lateralis* and *T. verticalis* were influenced by acclimation at different temperatures (temperature x species interactions $P < 0.001$; Table 5 - Bonferroni tests maximum $P = 0.014$; Supplementary Table 3) and conductivities (conductivity x species interaction $P < 0.001$; Table 5 - Bonferroni tests maximum = 0.026; Supplementary Table 3). For both species, freezing point was significantly influenced by both conductivity (*P* = 0.041; Table 5; Bonferroni tests maximum = 0.044; Supplementary Table 3) and acclimation temperature (*P* = 0.003; Table 5; Bonferroni tests maximum = 0.002; Supplementary Table 3). Mean SCPs also differed strongly between species (*P* = 0.001; Supplementary Table 3), being lower on average for *S. lateralis* (Bonferroni tests *P* = 0.001; Supplementary Table 3).

Overall, *S. lateralis* had a significantly lower SCP (i.e. greater tolerance to freezing) than *T. verticalis*. Such a significant effect was recorded at acclimation temperatures of 10 and 15 °C, but was reversed at 25 °C (Supplementary Fig. 2). At conductivities of 4 and 12 mS cm^{-1}, *S. lateralis* had a significantly lower SCP than the alien corixid (Supplementary Fig. 2). For *T. verticalis* only, post-hoc tests showed that SCP varied significantly both with temperature and conductivity, decreasing as temperature increased to 25 °C, and as conductivity increased to 18 mS cm^{-1} (Supplementary Table 3).

Table 5. Effect of acclimation temperature (T), acclimation conductivity (C) and species (Sp: *Trichocorixa verticalis* or *Sigara lateralis*) on corixid supercooling point (SCP) – General linear model.

Source	**SS**	**d.f**	**MS**	***F***	***P***
T	76.0	2	38.0	6.29	0.003
C	51.9	3	17.3	2.86	0.041
Sp	77.3	1	77.3	12.81	0.001
T × C	32.8	6	5.4	0.90	0.495
T × Sp	190.6	2	95.3	15.78	< 0.001
C × Sp	134.5	3	44.8	7.42	< 0.001

Sum of squares (SS); degrees of freedom (d.f.); mean square (MS), F-ratio (*F*), probability level (*P*).

Discussion

T. verticalis and *S. lateralis* differed strongly in their physiological responses to heating, cooling and freezing; a finding in agreement with (Chown et al., 2007a), who suggest that the form of physiological plasticity can be a key difference between invasive and native species. However, contrary to our expectations, *S. lateralis* generally outperformed *T. verticalis*, and appeared to possess a broader thermal tolerance range (*sensu* Calosi et al., 2010). Both temperature and conductivity influenced corixid thermal tolerance. However, the effect of exposure to different temperatures and conductivities varied between upper and lower limits for the two species examined.

Although the temperatures recorded for CT_{min} and SCP are below those encountered by corixids under field conditions in our study area, their relative values and plasticities allow us to compare the relative ability of *Trichocorixa* and *Sigara* to cope with cold. The minimum air temperature recorded at the Palacio de Doñana in 2012 was -6ºC and this matches the minimum value ever recorded in Doñana (February 1981; 2012), although temperatures below zero are not so unusual (http://www-rbd.ebd.csic.es/Seguimiento/mediofisico.htm). The CT_{max} values we recorded are ecologically very relevant, however, since the maximum air temperature often reaches 46ºC in July-August. Corixids concentrate in shallow water whose temperature can exceed that of the air in summer. For example, in ponds frequented by the study species, water temperature reached 39 ºC in May 2007 (authors' unpublished data), whilst air temperature in the same month did not exceed 34 ºC.

Critical thermal maximum

In terms of heat tolerance, the present study demonstrates that both species increase their CT_{max} in response to acclimation to a combination of high conductivity (18 mS cm^{-1}) and low temperature (10 ºC). Such an effect was also recorded by Verween et al., (2007), who found a trade-off between suboptimal temperature tolerance and high salinity in *Mytilopsis leucophaeata* (Mollusca, Bivalvia). Contrary to our initial expectations, acclimation to higher temperatures (25 °C) did not improve heat tolerance in either corixid species. From our findings it appears that both species possess a similar heat shock response at the higher temperature employed.

Insects express heat shock proteins (HSPs) in response to both cold and osmotic shock (Feder & Hofmann, 1999, Benoit et al., 2010). In *Drosophila*, exposure to low temperature results in heat shock protein upregulation when the animals are returned to higher temperatures (Chown et al., 2007b), suggesting that the interaction between low temperature exposure and acute heating can also increase heat resistance (Goto & Kimura, 1998). Both processes may operate in the corixids in our study, suggesting that although HSP expression can vary among and within species (Feder & Hofmann, 1999), they appear to exhibit similar capacities to regulate HSP production under laboratory conditions. Such a plastic thermotolerance response has already been reported in many organisms (Barua, Downs & Heckathorn, 2003) and here suggests that both corixids may use similar physiological mechanisms of acclimation when exposed to low temperatures and high salinity. On the other hand, the fact that both species did not elevate their heat tolerance after exposure to the higher temperature suggests that both species may maintain a high standing stock of HSPs in their cells. This mechanism often occurs in warm adapted organisms (Barua, Downs & Heckathorn, 2003), and suggests that new warmer conditions experienced in SW Spain by *T. verticalis* compared to its native range may have led to some physiological changes as an adaptation to the local conditions.

From our data, *S. lateralis* appears to be generally more heat tolerant than *T. verticalis.* It is possible that the differences in maximum heat tolerance observed in the present study are at least partly based on differences in body size between the two species. Body size-mediated thermal acclimatory responses of upper thermal limit have previously been reported for diving beetles (Sánchez-Fernández et al., 2010) and freshwater Crustacea (Mundahl & Benton, 1990), and could explain why the larger species *S. lateralis* showed a higher heat tolerance than *T. verticalis* here.

In general, warm adapted ectotherms possess great tolerance to heat (Stillman, 2003; Compton et al., 2007), but according to Stillman, (2003) they may have evolved this ability at the expense of their acclimatory capacity. Our results are in general agreement with Stillman's conclusion, since *S. lateralis* has a lower ability to acclimate CT_{max} in response to prior temperature exposure than *T. verticalis* (note how the alien shows greater magnitude of change in mean CT_{max} with temperature in Supplementary Table 1), despite having the highest absolute CT_{max} overall. The fact that *Trichocorixa* apparently has greater plasticity to heat than *S. lateralis* may make it better able to respond to sudden temperature shifts in nature, something which may favour its spread.

Critical thermal minimum

Whilst the native *S. lateralis* generally entered chill coma at lower temperatures, the response to acclimation conductivity was species specific. Whereas *S. lateralis* increased CT_{min} at lower conductivities, the opposite occurred for *T. verticalis*. Several previous studies have found effects of salinity on cold tolerance in other ectotherms, including *Nebrioporus* diving beetles, and fishes including the blackchin tilapia (*Sarotherodon melanotheron*) and the red drum (*Sciaenops ocellatus*) (Sánchez-Fernández et al., 2010; Stauffer, Vann & Hocutt, 1984), but see (Craig, Neill, & Iii, 1995). Doñana and surrounding areas such as the Odiel marshes are characterized by a Mediterranean subhumid climate with rainfall between late September and early April, hot and dry summers, and mild winters (Serrano et al., 2006). Salinity varies spatially and temporally, but many ponds and marshes in Doñana are oligohaline during the winter (Serrano et al., 2006). Given that *S. lateralis* overwinters as adults, our results suggest that its ability to better remain active at lower conductivities may reflect the ability to minimize energetic costs for osmoregulation during the winter season. However, such an adaptation for winter survival could bring a high cost for *S. lateralis* in terms of development, fecundity and longevity (Feder & Hofmann, 1999).

Cold hardiness and desiccation resistance are mechanistically linked, and one is thought to originally have developed from the other (Ring & Danks, 1994). Amongst *Drosophila* species, widespread species possess higher levels of resistance to both desiccation and cold (Kellermann et al., 2009). Furthermore, this lack of genetic limitation in resistance traits appears to help drive *Drosophila* distribution patterns. Thus, it is plausible that *T. verticalis* possesses such desiccation-inducible genes that are also induced by the desiccating effect of increases in ambient salinity. In response to osmotic stress at higher conductivities, these genes produce solutes that enhance cold tolerance (Sømme, 1999). In its native habitats, *T. verticalis* is considered to be a euryhaline insect (Hutchinson, 1993) and often occurs in brackish and saline waters (Rodríguez-Pérez et al., 2009). As with *S. lateralis*, *T. verticalis* overwinters as adults, but contrary to the native species, seems well adapted to overwinter in higher salinity waterbodies, like estuarine fish ponds (Rodríguez-Pérez et al., 2009; Van de Meutter, Trekels & Green, 2010). In this context, our results suggest that the osmoregulatory ability of *T. verticalis* may allow this alien to spend the cold season in saline wetlands, where it probably also achieves continuous reproduction and development. This would help explain its successful colonization of Doñana, especially its dominance in permanent, saline fish ponds (Rodríguez-Pérez et al., 2009; Van de Meutter et al., 2010).

We detected no effect of temperature of acclimation on CT_{min}, contrary to many previous studies on insects (e.g. Terblanche et al., 2005; Sisodia & Singh, 2010; Sánchez-Fernández et al., 2010). This absence of acclimatory ability shows limited temperature-dependent phenotypic plasticity for CT_{min} in our study species. Freezing winter temperatures are unusual in wetlands of southern Iberia, and these populations may not need well developed acclimatory abilities, which are known to have costs related to the severity of the stress (Rako & Hoffmann, 2006). In contrast, much colder winter temperatures are observed in the native range of *T. verticalis* along the east coast of North America (www.worldclim.org), and it would be interesting to compare native and invasive populations in this regard.

Supercooling point

Both corixid species are freeze-avoiding insects, as they both show pre-freeze mortality and the SCP represents their lower lethal limit to survival. Moreover, a decrease in SCP is likely to be part of their seasonal cold-hardening strategy (Lee, 1991). Different factors contribute to the enhancement of SCP capacity in insects, especially body size (Hahn, Martin & Porter, 2008). However, we didn't find an effect of intraspecific size variation in our study.

In the case of *T. verticalis,* cold hardiness was higher after acclimation to both higher temperatures and conductivities. This may result from physiological adjustments that probably involve heat protectant accumulation in response to high temperature and water loss regulation in response to osmoregulatory stress. As temperature increases, *T. verticalis* increase its heat tolerance, perhaps by HSP upregulation. The ability of HSPs to improve both heat and cold stress has been well documented in *Drosophila* species (Goto & Kimura, 1998), for reviews see (Feder & Hofmann, 1999), as has the influence of dehydration on insects' cold hardiness (Salt, 1961).

Since we did not observe any influence of either acclimation temperature or salinity on SCP in *S. lateralis*, it is possible that the native and exotic species differ fundamentally in their physiological ability to supercool. This lack of acclimatory ability of SCP in *S. lateralis* suggests that *T. verticalis* may in fact be better able to survive temperature and salinity fluctuations, despite the fact that it generally exhibited higher CT_{min} and SCPs than *S. lateralis*.

Implications for the invasion of *T. verticalis*

Overall, we found the native *S. lateralis* to be more thermally tolerant than the invasive *T. verticalis*, and our results may explain why *S. lateralis* remains dominant in freshwater ponds in the Doñana area, where *T. verticalis* is rare and has not been confirmed as a breeding species (Rodríguez-Pérez et al., 2009). However, our study supports the hypothesis that an ability to cope with environmental fluctuations, and a high resistance to salinity, favours the invasion of *T. verticalis* in the Mediterranean region. The tolerance of *T. verticalis* to both heat and freezing increases following exposure to high conductivities. The mean salinity of remaining natural wetlands in the Mediterranean basin is much higher than in northern Europe (Declerck et al., 2005; Moss et al., 2009), partly because freshwater wetlands have been drained more extensively (Green et al., 2002). Under a scenario of further climatic warming, greater evapotranspiration rates are likely to promote further increases in salinity (Moss et al., 2009), and as a consequence, species able to cope with higher salinities may benefit from ongoing global change. The ability of *T. verticalis* to survive and reproduce in waters of relatively high conductivity during winter may be central to its success. The regular droughts occurring in the Mediterranean region mean that some winters see so little rain that many freshwater marshes do not flood, and in regions such as Doñana, this leaves water only in brackish fish ponds or coastal salt-pans which are now dominated by *T. verticalis* (Kloskowski et al., 2010). Our results suggest that *T. verticalis* has higher cold tolerance than *S. lateralis* in such habitats, a factor which is likely to contribute to its overwinter survival and reproduction. Saline waters may act as sources of the invasive *T. verticalis* for the surrounding freshwater habitats in Doñana and elsewhere, and its broad salinity tolerance and ongoing salinization of aquatic habitats may play important roles during the invasion.

Plasticity is a recognized characteristic of good invaders (Richards et al., 2006; Ghalambor et al., 2007) and the thermal physiology of *T. verticalis* is consistent with this pattern. The greater range of mean responses recorded across our 12 experimental treatments in *T. verticalis* compared to *S. lateralis* (4.56 vs 3.46 ^{o}C for CT_{max}; 3.04 vs 2.68 ^{o}C for CT_{min}; 7.65 vs 3.89 ^{o}C for SCP) all point to greater plasticity in the invader. In addition to its physiological abilities, life history characteristics may play a central role in the invasion success of *T. verticalis*. According to Sol et al. (2012), successful invaders can face the ecological pressure posed by the newly invaded environment by allocating reproductive efforts over several breeding events. *T. verticalis* has multiple generations a year in permanent fish ponds in Doñana (authors unpublished data), whereas *S. lateralis* is bivoltine (Cianferoni, 2009). Whilst there are limited data on the life-history of native populations of *T. verticalis* in the Americas, it appears that the warmer climate of the Mediterranean area may have allowed this species to switch to reproducing throughout the year, as suggested in previous studies (Van De Meutter et al., 2010). Such responses can occur rapidly following invasion. Japanese populations of the faall webworm (*Hyphantria cunea*, Lepidoptera) have shifted from being bivoltine to trivoltine in 25 years when exposed to new environmental conditions (Gomi, 2007). In *T. verticalis*, the ability to reproduce throughout the year, together with an apparently greater plasticity to heat, cold and salinity could facilitate its survival in the face of new environmental conditions, and indeed facilitate its spread as climate change proceeds.

Finally, whilst *T. verticalis* occurs in sympatry with the native *S. lateralis* in Spain (Rodríguez-Pérez et al., 2009), it also appears to overlap the salinity niche of some halophilic European corixids such as *S. selecta* (Fieber, 1848) and *S. stagnalis* (Leach, 1817) (Van de Meutter, Trekels & Green, 2010). Future research should address possible interactions with these other species, since the outcomes of these encounters may not be identica.

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Supplementary Materials

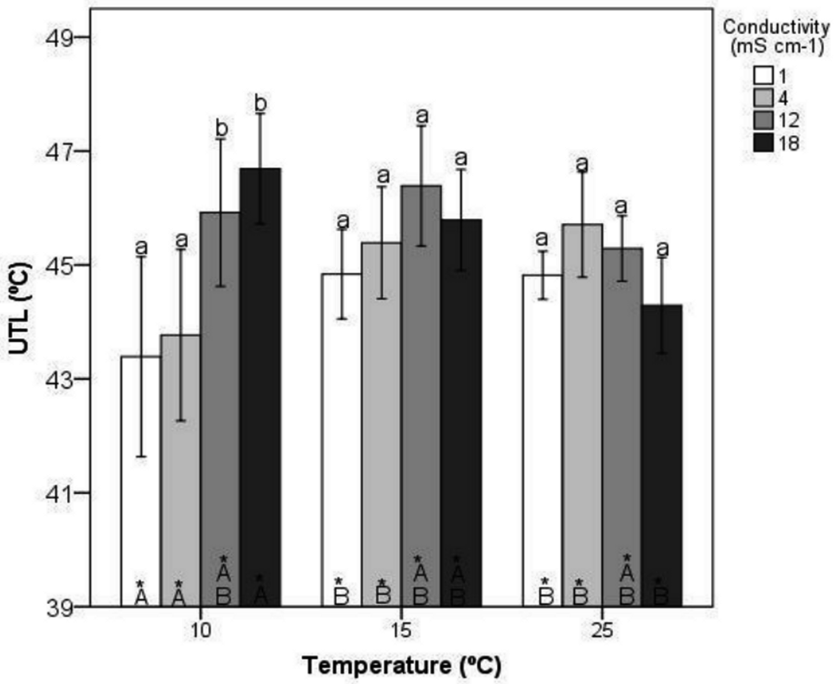

Figure 1. Interactive effect of temperature and conductivity on mean CTmax. Histograms are mean ± SE critical thermal maximum (CT_{max}) of *Sigara lateralis* and *Trichocorixa verticalis* acclimated to different temperatures (10, 15 and 25 °C) and conductivities (1, 4, 12, 18 mS cm^{-1}). Significantly different means ($P < 0.05$) between different acclimation temperatures measured at the same acclimation conductivity are indicated by different capital letters inside the histograms, whereas significantly different means measured at different conductivities at the same acclimation temperature are indicated by different lower case letters above or below the histograms (according to Estimated Marginal Mean test with Bonferroni correction).

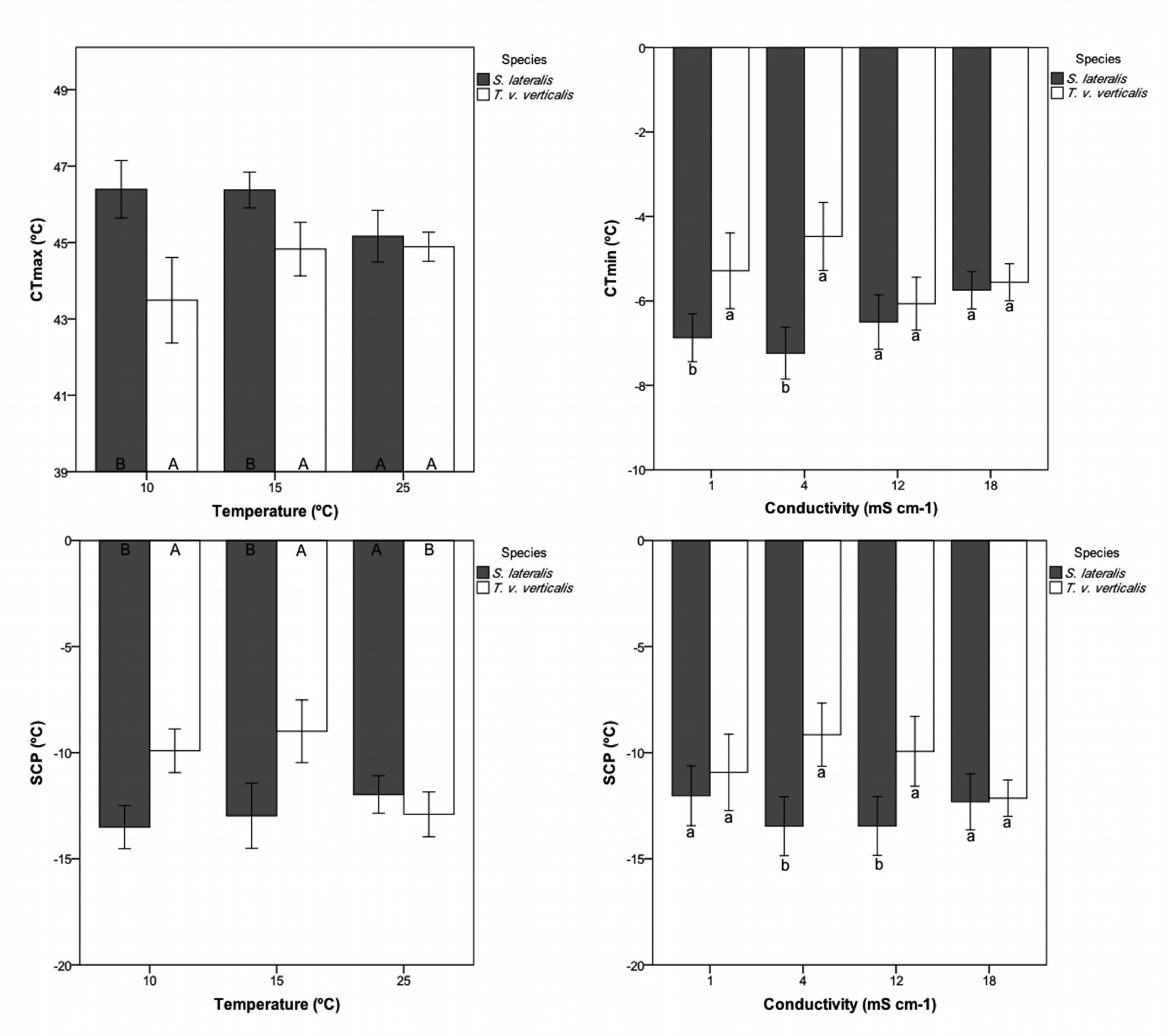

Figure 2. Thermal limit and freezing point differences between *Trichocorixa verticalis* and *Sigara lateralis***.** Histograms of mean ± SE critical thermal maximum (CT_{max}), critical thermal minimum (CT_{min}) and supercooling points (SCP) of *Sigara lateralis* and *Trichocorixa verticalis* acclimated to different temperatures (10, 15 and 25 °C) and conductivities (1, 4, 12, 18 mS cm^{-1}), according to linear model output. Significantly different means between species ($P < 0.05$) measured at different acclimation temperatures are indicated by different capital letters inside the histograms, whereas significantly different means between species measured at different conductivities are indicated by different lower case letters above or below the histograms (according to Estimated Marginal Mean test with Bonferroni correction).

Table 1. Significantly different mean CT_{max} (Estimated Marginal Means tests with Bonferroni correction) from Table 2 according to acclimation temperature (T), acclimation conductivity (C), species (Sp: *Trichocorixa verticalis* or *Sigara lateralis*) and sex (1= male; 2 = female). These tests refer to partial effects from the final model.

conductivity vs temperature

C	(I)T	(J)T	Mean Difference (I-J)	Std. Error	Sig.
1	10	15	-1.45	0.59	0.049
		25	-1.49	0.59	0.040
4	10	15	-1.68	0.59	0.016
		25	-2.00	0.59	0.003
18	10	25	2.26	0.59	0.001

temperature vs conductivity

T	(I)C	(J)C	Mean Difference (I-J)	Std. Error	Sig.
10	1	12	-2.39	0.59	0.001
		18	-3.23	0.59	<0.001
	4	12	-2.08	0.59	0.004
		18	-2.92	0.59	<0.001

temperature vs species

T	(I)Sp	(J)Sp	Mean Difference (I-J)	Std. Error	Sig.
10	*Sl*	*Tvv*	2.69	0.43	<0.001
15	*Sl*	*Tvv*	1.33	0.43	0.002

species vs temperature

Sp	(I)T	(J)T	Mean Difference (I-J)	Std. Error	Sig.
Sl	10	25	1.09	0.42	0.035
	15	25	1.10	0.42	0.031
Tvv	10	15	-1.37	0.42	0.004
		25	-1.46	0.42	0.002

conductivity

(I) C	(J) C	Mean Difference (I-J)	Std. Error	Sig.
12	1	1.44	0.34	< 0.001
18	1	1.17	0.34	0.006

species

(I) Sp	(J) Sp	Mean Difference (I-J)	Std. Error	Sig.
Sl	*Tv*	1.39	0.25	< 0.001

sex

(I) Sex M	(J) Sex F	Mean Difference (I-J)	Std. Error	Sig.
1	2	-0.69	0.31	0.030

Table 2. Significantly different mean CT_{min} (Estimated Marginal Means tests with Bonferroni correction) from Table 4 according to acclimation conductivity (C) and species (Sp: *Trichocorixa verticalis* or *Sigara lateralis*). These tests refer to partial effects from the final model.

conductivity vs species

C	(I) Sp	(J) Sp	Mean Difference (I-J)	Std. Error	Sig.
1	*Sl*	*Tv*	-1.58	0.43	< 0.001
4	*Sl*	*Tv*	-2.76	0.43	< 0.001

species vs conductivity

Sp.	(I) C	(J) C	Mean Difference (I-J)	Std. Error	Sig.
Sl	4	18	-1.49	0.43	0.006
Tv	12	4	-1.59	0.43	0.003

species

(I) Sp.	(J) Sp.	Mean Difference (I-J)	Std. Error	Sig.
Sl	*Tv*	-1.24	0.22	< 0.001

Table 3. Significantly different mean SCPs (Estimate Marginal Means tests with Bonferroni correction) from Table 5 according to acclimation temperature (T), acclimation conductivity (C) and species (Sp: *Trichocorixa verticalis* or *Sigara lateralis*). These tests refer to partial effects from the final model.

temperature vs species

T	(I) Sp.	(J) Sp.	Mean Difference (I-J)	Std. Error	Sig.
10	*Sl*	*Tv*	-3.59	0.77	< 0.001
15	*Sl*	*Tv*	-3.98	0.77	< 0.001
25	*Sl*	*Tv*	2.37	0.95	0.014

species vs temperature

Sp	(I) T	(J) T	Mean Difference (I-J)	Std. Error	Sig.
Tv	25	10	-4.43	0.95	< 0.001
		15	-5.35	0.95	< 0.001

species vs conductivity

Sp	(I) C	(J) C	Mean Difference (I-J)	Std. Error	Sig.
Tv	18	1	-3.38	1.15	0.026
		4	-5.15	1.15	< 0.001
		12	-4.37	1.15	0.002

conductivity vs species

C	(I) Sp	(J) Sp	Mean Difference (I-J)	Std. Error	Sig.
4	*Sl*	*Tv*	-4.31	0.89	< 0.001
12	*Sl*	*Tv*	-3.51	0.89	< 0.001

conductivity

(I) C	(J) C	Mean Difference (I-J)	Std. Error	Sig.
4	18	2.00	0.73	0.044

temperature

(I) T	(J) T	Mean Difference (I-J)	Std. Error	Sig.
25	15	-2.17	0.61	0.002

species

(I) Sp	(J) Sp	Mean Difference (I-J)	Std. Error	Sig.
Sl	*Tv*	-1.73	0.48	0.001

Chapter 2

Niche partitioning between invasive and native corixids (Hemiptera, Corixidae) in south-west Spain.

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In Review in *Aquatic Sciences*

Abstract

Trichocorixa verticalis verticalis, a North American water boatman, is the only alien corixid in European fresh waters. It has rapidly spread, becoming the dominant corixid in and around the Doñana protected area (SW Spain). Its high abundance and similar morphology to native corixids suggest that *T. verticalis* may impact on them through competition for food. Here we used stable isotopes of nitrogen (N) and carbon (C) and Bayesian analytical tools to investigate niche partitioning between *T. verticalis* and the natives *Sigara lateralis* and *S. scripta* through a combination of experimental and field data. Species sampled from permanent ponds and laboratory aquaria could be separated based on their isotopic values ($\delta^{15}N$ and $\delta^{13}C$). *S. lateralis* consistently showed higher $\delta^{15}N$ values than *T. verticalis*, suggesting that the invasive species may be feeding at a lower trophic position and relying more on herbivory than its native competitors. This was particularly true for the *T. verticalis* nymph stage, which showed depleted $\delta^{15}N$ values compared to adults, indicating ontogenetic dietary shifts. In contrast, native corixids and the invasive species showed similar isotopic compositions in temporary habitats at different stages in the flooding-desiccation cycle, suggesting some degree of niche overlap and a slight reduction in trophic level for *S. lateralis* when inhabiting ephemeral systems. The combination of experimental and field data has been useful to depict trophic interactions during a biological invasion and stable isotopes provided insights into the trophic ecology of this invasive species and into mechanisms that facilitate species co-existence in the invaded range.

Introduction

Invasion by exotic species is a global ecological and conservation problem, causing community change and species extinctions worldwide (Crowl et al., 2008; Vilà et al., 2010; Hermoso et al., 2011; Strayer, 2012). The impact caused by biological invasions can range from undetectable to dramatic (Edelaar & Tella, 2012) depending partly on how invasive species traits, including behaviour or physiology, match those of natives in the invaded community (Strauss et al., 2006). Several studies have related traits such as competitive ability for food and/or space to invasion success, as invasive species are often considered superior competitors (Levine et al., 2003; Vilà & Weiner, 2004).

When competition occurs, the degree of resource/niche overlap among invasive and native species largely determines species exclusion or co-existence (De Roos et al., 2008). Niche similarity between species can cause the strongest impact on inferior competitors (Dick, 2008) through behavioural alterations (i.e., shifts in habitat use and foraging niche) or species exclusion and extinction (Begon et al., 1996; Simon & Townsend, 2003). In contrast, niche differentiation, in which competing species specialise on distinct resources (resource partitioning) or exploit the same resources at different places or times (spatial or temporal niche partitioning), favours species co-existence (Koch, 1974; Tilman, 1982; Chesson, 2000).

The outcome of species relationships, however, is context specific (Chesson, 2000). For example, competitive interactions, within and between species, may be reversed among habitats that differ in productivity and resource diversity (Tobler, 2008). In recent years, stable isotopes and novel quantitative metrics have been proved to be valuable tools in identifying trophic niche characteristics and trophic interactions among species (Olsson et al., 2009; Zambrano et al., 2010; Piscart et al., 2011; Eloranta et al., 2013; Jackson et al., 2013). Stable isotopes are especially powerful because they integrate information over long time periods (Bearhop et al., 2004; Olsson et al., 2009; Atkinson et al., 2010), and do not have the biases of gut analysis (Clarke et al., 2005; Araújo et al., 2007). The latter is not always easy to apply, particularly in small species, given the difficulties of identifying material in their guts. However, measures of niche dimensions at a population level can bring misinterpretation if the inherent variability of consumers (i.e. individual physiology) and the spatial isotopic variability in the diet are not taken into account (Jackson et al., 2011).

Freshwaters habitats have proportionally more invaders than terrestrial systems (Vitousek et al., 1997). Nonetheless, for the majority of these invaders the effects on the invaded systems are largely unknown. Exceptions include the zebra mussel *Dreissena polymorpha,* the mosquitofish *Gambusia holbrooki* and the crayfish *Procambarus clarkii* (Pimentel et al., 2005; Caiola & Sostoa, 2005; Savini et al., 2010). The North American boatman

Trichocorixa verticalis verticalis (Fieber, 1851) (Heteroptera, Corixidae) is a successful invader in south-west Iberian Peninsula. It has established populations at several sites, including the Doñana wetland complex, where it is the dominant breeding corixids in saline wetlands (Rodríguez-Pérez et al., 2009; Van de Meutter et al., 2010). It was first recorded in Algarve (Portugal) in 1997 and has since become widespread in southern Portugal (Sala & Boix 2005), south-west Spain (Rodríguez-Pérez et al., 2009; Van de Meutter, Trekels & Green, 2010) and Morocco (L'Mohdi et al., 2010). It is predicted to spread widely across Europe and the Mediterranean region in the future (Guareschi et al., 2013).

Given the voracity of *T. verticalis* in its native range (Wurtsbaugh, 1992) and the morphological similarity of piercing and sucking mouthparts within Hemiptera, we can expect a high feeding impact of the invasive corixid on native corixids, especially *Sigara lateralis* and *S. scripta,* which are often found to co-occur in the invaded range. Furthermore, the wide spectrum of potential food used by *T. verticalis,* including zooplankton, filamentous algae and dipteran larvae (Kelts, 1979; Simonis, 2013) suggests that other important ecological interactions might occur. In its native range, for example, *T. verticalis* is likely to cause changes in ecosystem processes via trophic cascades (Wurtsbaugh, 1992; Simonis, 2013). Nonetheless, there is no previous information about trophic relationships between *T. verticalis* and native co-occurring corixid species, and little is known about its trophic ecology in the invaded areas.

In this study we applied stable isotopes ($\delta^{15}N$ and $\delta^{13}C$) and Bayesian tools to experimental and field data to assess the potential of *T. verticalis* to compete with native species in the invaded range. We are not aware of any previous studies that compare stable isotopic variability between experimental and wild populations. This approach offers the possibility to examine the trophic relationships between species removing biases that may be related to spatial or age variability. Specifically, we examine niche partitioning among *T. verticalis* and native corixids in permanent and temporary waterbodies in Doñana, so as to understand changes between their trophic relationships and habitat variability. In addition, we aim to further our understanding of the trophic ecology of *T. verticalis* where it has stable reproductive populations within Doñana, and its potential impact upon the invaded aquatic systems. Because *T. verticalis* was often found in syntopy with native corixids in temporary waterbodies, we hypothesised that niche differentiation through resource partitioning facilitates their co-existence. However, we expected a different outcome of species interactions in stable saline permanent ponds where *T. verticalis* dominates. Finally, we discuss the implications of our results for conservation of native corixids and prey communities.

Material & Methods

Laboratory experiment

To reduce the effect of potentially confounding factors other than niche partitioning that may cause isotopic differences between corixids (e.g. age-related dietary differences or spatial segregation), a laboratory experiment was conducted between August and September 2012 at the Doñana Biological Station-CSIC. Aquaria (6 L, 27x17x18 cm) were filled with sediments and water collected from the FAO pond within Doñana National Park, where *T. verticalis* and *S. lateralis* were co-existing. Salinity in aquaria was less than 1 ppt. One plastic circular jar lid (~9 cm diameter) was added to each aquarium as a substrate for periphyton (i.e. epilithon) growth. Aquaria were maintained with a 12h:12h dark/light regime until flora and fauna from egg and seed banks within pond sediment colonized them. After three weeks, we added V instar corixid nymphs to each aquarium. These nymphs had been reared in the laboratory from the egg stage. To collect eggs, adults of *T. verticalis* and *S. lateralis* originating from the FAO pond were placed separately in aquaria containing 5 X 5 cm of 500 µm plastic mesh (known from previous observations to be a preferred substrate for egg laying). Meshes were later carefully removed and placed in separate aquaria until eggs hatched. Nymphs were then fed with lyophilized microalgae (*Tetraselmis chuii*) until they reached the V instar.

We added 15 or 20 nymphs of each species in the same aquarium, with three replicates. Live chironomid larvae from the FAO pond were added one day after the experiment started. After 16-23 days, adult corixids and resources were removed and processed for stable isotope analysis. A sufficient number of nymphs became adults (after 16-23 days) in only two aquaria (AQ1, AQ2) and were thus used for stable isotope determinations. This time is considered enough to capture the overall isotope change (Gratton & Forbes 2006), which is also mirrored by the rapid chitin turnover (Schimmelmann, 2011).

Field study

Corixids and their potential food resources were collected during June-July 2011 and February 2012 from areas within the Doñana wetland complex (Fig. 1). Five permanent ponds, located in Veta la Palma estate within Doñana Natural Park (hereafter VLP), were sampled twice during summer (July) and winter (February). Four temporary waters were sampled only once (June). These sites were inside Doñana National or Natural Park and included two ponds (local names 9N3PP and AC3, hereafter T1 and T2), one section of a seasonal stream (Entremuros, hereafter T3), and one shallow lake (Lucio del Lobo, hereafter T4).

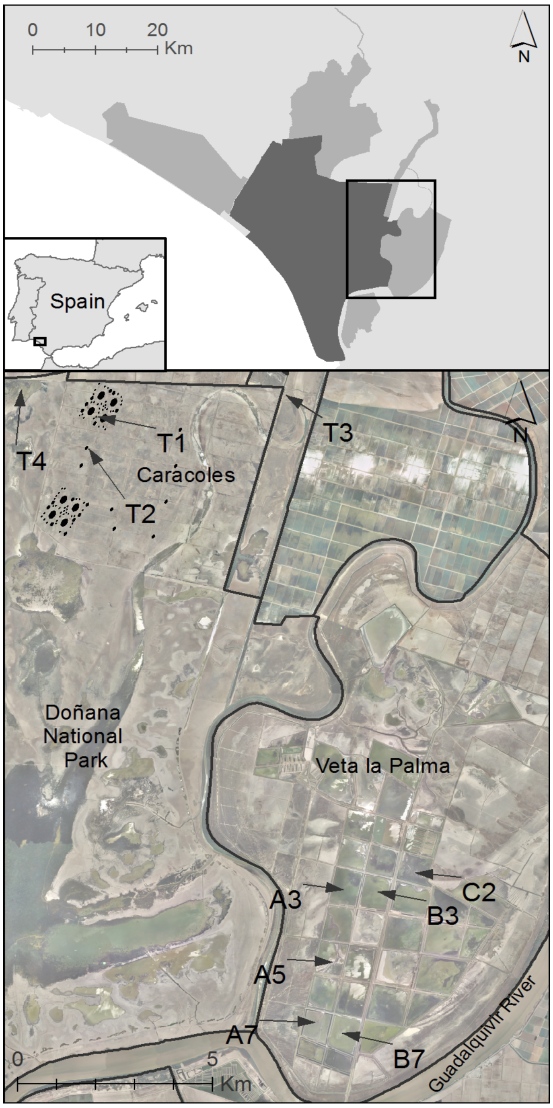

Figure 1. Map of the sampling sites in Doñana. The dark grey area in the upper figure indicates Doñana National Park and the light grey area indicates Doñana Natural Park. Six permanent ponds are identified in Veta la Palma estate, and four temporary sites are identified in and around the Caracoles estate.

Water salinities varied spatially and temporally, from 8.7 and 19.8 psu in summer to 10.2 and 18.3 psu in winter in permanent ponds, and between 5.4 and 15.9 psu in temporary sites (see Supplementary Table 1). Details of VLP and its aquatic invertebrate community are given by Rodriguez-Pérez & Green, (2012). The temporary sites were described by Frisch et al. (2012).

In general, the alien *T. verticalis* dominates in permanent, saline habitats in VLP, whereas native corixids dominate in temporary sites of lower salinity in Doñana National Park (Rodríguez-Pérez et al., 2009; Van de Meutter et al., 2010). As native corixids, we selected *Sigara lateralis* and *S. scripta* because they represent the most frequent and abundant corixids found in syntopy with *T. verticalis*. Corixid size differed between species, with *S. lateralis* (mean total length ± SE, 4.73 ± 0.25 mm, *n* = 103,) the larger species, followed by *S. scripta* (4.39 ± 0.25 mm, *n* = 93) and *T. verticalis* (4.16 ± 0.32 mm, *n* = 111).

When possible, 20 adults of each species, balanced for sex, were collected. Nymphs (III, IV and V instars) of *T. verticalis* were only obtained in February from permanent ponds. No adult corixids were found in two of the five sampled permanent ponds in winter. Details of sampling methods for flora and fauna in permanent and temporary sites are summarized in Supplementary Table 2. With the exception of periphyton (i.e. epiphyton), potential resources were always collected at different locations within permanent ponds because they were also used for a broader study of pond food webs. In the case of temporary waterbodies, corixids and sources were collected in the same area (ca. 20 m^2) within the site.

After collection, samples were kept inside plastic containers filled with a minimal volume of water, placed within a portable freezer and transported to the laboratory. Once at the laboratory, suspended particulate organic matter (POM), phytoplankton and seston were obtained by filtering a known volume of water (pre-filtered through a 100 µm mesh) onto Whatman GF/F glass fiber filters (ø= 47 mm) under vacuum. Filters were then packed into aluminium paper and frozen. The periphyton (i.e. epiphyton) suspension (Supplementary Table 2) was filtered through Whatman GF/F glass fiber filters in the same way as seston/POM. Flora and fauna were rinsed with distilled water and put in Eppendorf tubes (1.5 ml) or in plastic bags before being frozen. Samples were frozen as soon as possible using a liquid nitrogen tank and stored until processing. All samples were thawed and oven-dried at 50°C for 24h before stable isotope analysis.

POM, phytoplankton, seston and periphyton (i.e. epiphyton) were exfoliated from the glass filters and pulverized. POM filters were fumigated with concentrated HCl and subsequently re-dried. VLP sediments were sequentially acidified with 0.1M HCl to remove carbonates, then oven-dried, while sediments from temporary sites were sub-sampled in two groups. For δ^{13}C analyses, subsamples were washed with diluted HCl to remove carbonates and redried. δ^{15}N was measured on untreated samples, as HCl treatment has been reported to affect δ^{15}N values (Bunn et al., 1995). In both cases, sediments were considered as a proxy for periphyton (i.e. epipelon), however, nitrogen estimates were conservative for VLP sediments.

Adult corixids from VLP were analyzed whole, while adults minus one leg were analysed from individuals from temporary sites. *T. verticalis* nymphs and chironomids were pooled for analyses, as samples of multiple individuals. Dry samples were homogenised, weighed and packed into tin capsules before analysis.

Stable isotope analysis

Isotopic analyses of carbon and nitrogen contents were carried out at the UC Davis Stable Isotope Facility (University of California, Davis) and in the Laboratory of Stable Isotopes at EBD-CSIC (www.ebd.csic.es/lie/index.html). Samples from VLP were analysed using a PDZ Europa Scientific Roboprep elemental analyser in line with a PDZ Europa Hydra 20/20 isotope ratio mass spectrometer (Crewe, UK) at UC Davis, while samples from laboratory experiment and temporary sites (about 0.9–1 mg) were combusted using a continuous flow isotope-ratio mass spectrometry system (Europa Scientific, UK) by means of a Carlo Erba 1500 N C elemental analyser interfaced with a Delta Plus CL isotope ratio mass spectrometer at the EBD-CSIC.

All isotope results are expressed in δ-notation as parts per thousand (‰) deviation, referred to international standards for nitrogen (i.e., Air) and carbon (i.e., Vienna Pee Dee Belemnite) as defined by the equation: $\delta^{13}C$, $\delta^{15}N = [(R_{sample} / R_{reference}) - 1] \times 10^3$, where $R = {}^{13}C/{}^{12}C$ for carbon and ${}^{15}N/{}^{14}N$ for nitrogen. The long-term standard error (based on replicate analyses of standard reference material) for samples measured at UC Davis was ± 0.2 and ± 0.3 for $\delta^{13}C$ and $\delta^{15}N$, respectively and for samples measured at LIE was ± 0.1 and ± 0.2 for $\delta^{13}C$ and $\delta^{15}N$, respectively. See Supplementary Table 3 for the standard reference material used at each laboratory.

A total of 505 corixids (277 *T. verticalis*, 160 *S. lateralis* and 68 *S. scripta*) were analyzed from field studies, and 34 (18 *T. verticalis* and 16 *S. lateralis*) from laboratory experiments.

Statistical analyses

Non parametric Kruskal–Wallis or Mann-Whitney U tests were used to test for differences in $\delta^{13}C$ and $\delta^{15}N$ values among species, or between *T. verticalis* adults and nymphs. These tests were conducted within each waterbody (permanent or temporary) or aquarium. Significance levels of non-parametric post hoc tests were Holm-Bonferroni corrected, when appropriate.

Kruskal–Wallis or Mann-Whitney U tests were used to test for spatial (among ponds) differences in isotopic values for species and food sources, and seasonal differences in *T. verticalis* isotopic composition and food sources. These analyses were conducted using the ponds common to each season (3 permanent ponds) and the food sources common to each pond (i.e. sediments and POM).

Estimated trophic position within habitats

Within each studied aquarium or waterbody (permanent and temporary) we constructed the mixing polygon of potential sources and corrected consumers for trophic enrichment using the values reported by McCutchan et al. (2003): $\delta^{13}C$ 0.5‰ and $\delta^{15}N$ 2.3‰ for corixids with mixed diet. We also estimated the relative mean trophic position of corixids by using phytoplankton values (laboratory and temporary sites) or POM (which is considered as a proxy of phytoplankton in permanent ponds) as baselines, and assuming a mean trophic enrichment of 2.3‰ for $\delta^{15}N$ between corixids and their food sources following McCutchan et al. (2003). POM and phytoplankton were selected as baseline because these were the resources common to each studied system. Given the larger error associated with the use of primary producers (Vander Zanden & Rasmussen 2001), absolute values for these estimates must be only considered as a proxy of their actual trophic position. However, since corixid $\delta^{15}N$ values were corrected for site-specific $\delta^{15}N$ baselines, these estimates were still useful for capturing differences in trophic positions across systems.

Niche width and trophic structure

To visualise how corixid populations were separated, or overlapped each other, in $\delta^{13}C$-$\delta^{15}N$ bi-plot space within each system, we calculated Standard Ellipses Areas corrected for sample size (SEA_c expressed in ‰^{2}) following Jackson et al. (2011) and using the Bayesian Stable Isotopes Ellipses (SIBER) in the SIAR package in R. Standard ellipses contain ca. 40% of the data, and were obtained from the variance and covariance matrix of x ($\delta^{13}C$) and y ($\delta^{15}N$) data. Therefore, an ellipse represents the core isotopic niche for each species and is a proxy of the richness and evenness of resources consumed by the population (Bearhop et al., 2004). When possible the SEAc was used to calculate the degree of standard ellipse overlap, between species within each individual pond, i.e. the percentage of area that overlap relative to the total isotopic niche occupied by the two species, and the Bayesian estimates of SEA ($SEA_{B,}$ bootstrapped n=10000) were generated to test for significant differences between corixid isotopic niche widths by comparing their confidence intervals (Jackson et al., 2012). The SEAc method is equivalent to the convex hull area proposed by Layman et al. (2007), and has the advantage of avoiding any bias when a minimum of 20 individuals is used, as we often did in this study (Brind'Amour & Dubois 2013). However, slightly lower sample sizes are also less biased if the variability in populations is low (Syväranta et al., 2013). Jackson et al. (2011) further discussed the relationship between SEA, SEAc and SEA_B. Niche width comparisons were only possible when the spatial variation in corixid isotopic composition could be dismissed (Cummings et al., 2012). We tested for distance between ellipse centroid locations (D) following the methodology of Turner et al. (2010). Two centroids are considered to occupy different locations if the distance between them is significantly greater than zero (Turner et al., 2010). All these metrics and test statistics were performed with R (v 2.15.1), using the package SIAR (Jackson et al., 2011) and the scripts provided by Turner et al. (2010).

Results

Stable Isotopes

T. verticalis was significantly more depleted in $\delta^{15}N$ values than *S. lateralis* in each aquarium (Mann-Whitney U tests $P < 0.05$), but $\delta^{13}C$ values differed significantly only in AQ2 (mean isotopic differences between species: 0.3‰ for $\delta^{13}C$ and 3.6‰ for $\delta^{15}N$ at AQ1; 1.1‰ for $\delta^{13}C$ and 1.7‰ for $\delta^{15}N$ at AQ2) (Fig. 2).

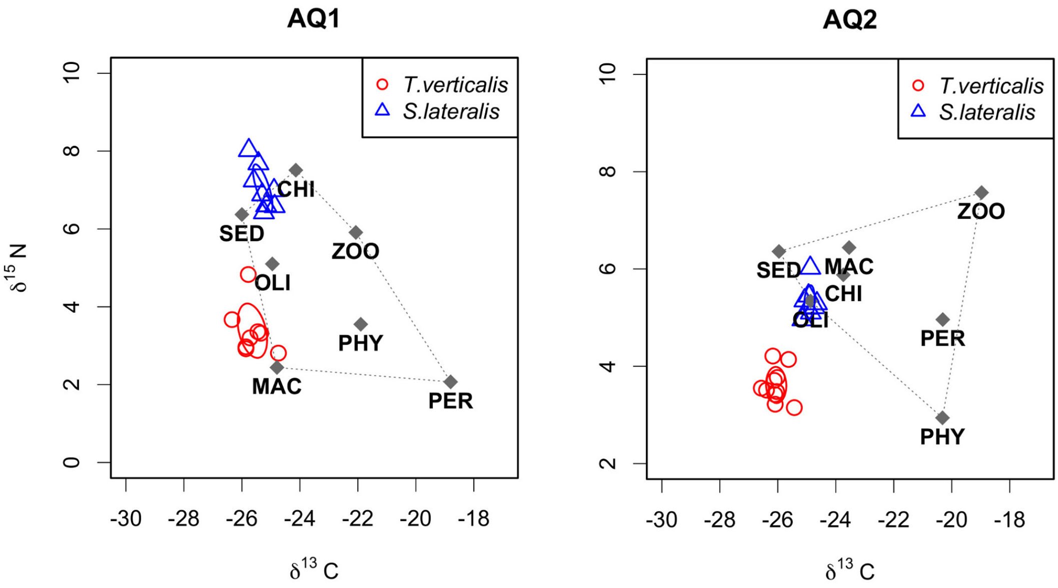

Figure 2. Dual-isotope food web diagram of aquaria including Standard ellipses showing differences in *Trichocorixa verticalis* and *Sigara lateralis* isotope values. Food web components: CHI=chironomidae; PER= periphyton; PHY= phytoplankton; OLI= oligochaeta; MAC= macrophytes; SED= sediments; VGR= vegetation roots; VGD= vegetal detritus; ZOO=zooplankton. Dashed lines represent the mixing polygon circumscribed by the isotopic signature of several resources (mean $\delta^{13}C$ and δ^{15}). Corixid $\delta^{13}C$ and $\delta^{15}N$ values were corrected for trophic enrichment.

T. verticalis and *S. lateralis* showed significant differences in both $\delta^{13}C$ and $\delta^{15}N$ values within each permanent pond where they co-existed ($P < 0.001$). On average, *S. lateralis* was more ^{13}C-depleted and ^{15}N-enriched than *T. verticalis* (mean isotopic differences between species: 4.5‰ for $\delta^{13}C$ and 1.3‰ for $\delta^{15}N$ at A3; 2.5‰ for $\delta^{13}C$ and 3.2‰ for $\delta^{15}N$ at B3) (Fig. 3). We did not find any spatial variation among the ponds where both species co-existed for *T. verticalis* ($P > 0.05$), but a significant difference in $\delta^{13}C$ ($P = 0.02$) was found for *S. lateralis*. However, we did not find any significant differences in the isotope values of the common sources between ponds ($P > 0.05$).

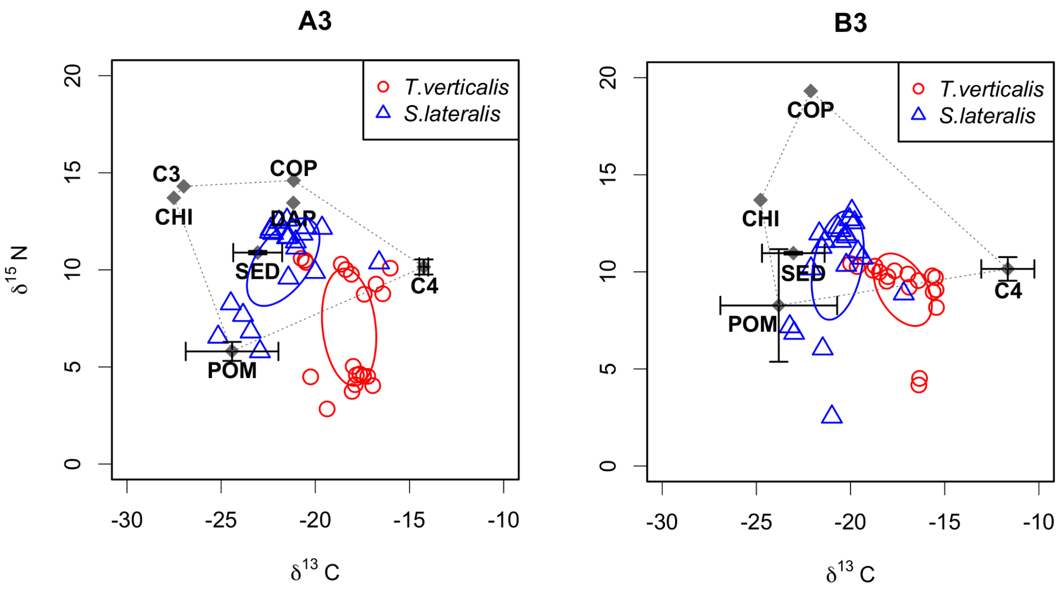

Figure 3. Dual-isotope food web diagram of two permanent ponds showing the Standard ellipse area (SEAc) of *Trichocorixa verticalis* and *Sigara lateralis.* CHI=chironomidae; COP= copepods; POM= particulate organic matter, PHR= *Phragmites* sp.; SED= sediments; RUP = *Ruppia* sp.; SPR = *Spartina* sp.+*Ruppia* sp.; PPE =*Potamogeton pectinatus.* Corixid $\delta^{13}C$ and $\delta^{15}N$ values were corrected for trophic enrichment.

T. verticalis adults were more ^{15}N-enriched than nymphs at permanent ponds B7 (0.2‰) and A5 (1.0‰) ($P < 0.05$), and significantly more ^{13}C-depleted (2.4‰) than nymphs at pond B7 ($P = 0.006$). No differences were found between adults and nymphs at pond A7 (Supplementary Fig. 1). Adults of *T. verticalis* showed significant spatial differences in both $\delta^{13}C$ and $\delta^{15}N$, but we did not find any spatial differences in isotope values for sediments or POM ($P > 0.05$). Strong seasonal differences were also found for $\delta^{13}C$ and $\delta^{15}N$ values ($P < 0.05$). In winter, *T. verticalis* was overall more ^{13}C-depleted (mean =-18.4‰) but ^{15}N-enriched (mean = 11.4‰) than in summer (mean $\delta^{13}C$ = -16.7‰; mean $\delta^{15}N$ = 9.7‰). We did not find any seasonal differences in $\delta^{13}C$ for sediments or POM ($P > 0.05$), but there was a significant seasonal difference in $\delta^{15}N$ value in each source ($P < 0.05$). In winter, values for sediments and POM were more ^{15}N-depleted than in summer (sediments mean = 8.06 vs. 11.20; POM mean = 2.52 vs. 6.16) (Supplementary Fig. 2).

Interspecific differences in isotopic values were more pronounced among temporary sites (Fig. 4). *T. verticalis* was significantly ^{13}C-enriched at T1 relative to *S. lateralis* and *S. scripta*, but the opposite was observed at T2 (Holm-Bonferroni test $P < 0.05$). The alien species was significantly enriched in ^{13}C relative to *S. lateralis* at T4 ($P < 0.001$), but depleted in ^{13}C compared to *S. scripta* at T3 ($P = 0.01$, Table 1). *T. verticalis* was significantly depleted in ^{15}N compared to both native species at T1 ($P < 0.001$, Table 1) and to *S. lateralis* at T4 ($P = 0.03$).

Strong spatial differences between temporary sites were also found for *T. verticalis* and *S. scripta* in δ^{13}C values (Kruskal-Wallis test $P < 0.001$) and in both δ^{13}C and δ^{15}N values for *S. lateralis* ($P < 0.001$). Both δ^{13}C and δ^{15}N values of sediments and phytoplankton also differed among sites ($P < 0.05$). Spatial differences were found in δ^{15}N for periphyton ($P = 0.03$) and in δ^{13}C for chironomids ($P = 0.01$), but we did not find any isotopic difference for *Scirpus* spp. among sites ($P > 0.05$) (Supplementary Table 4).

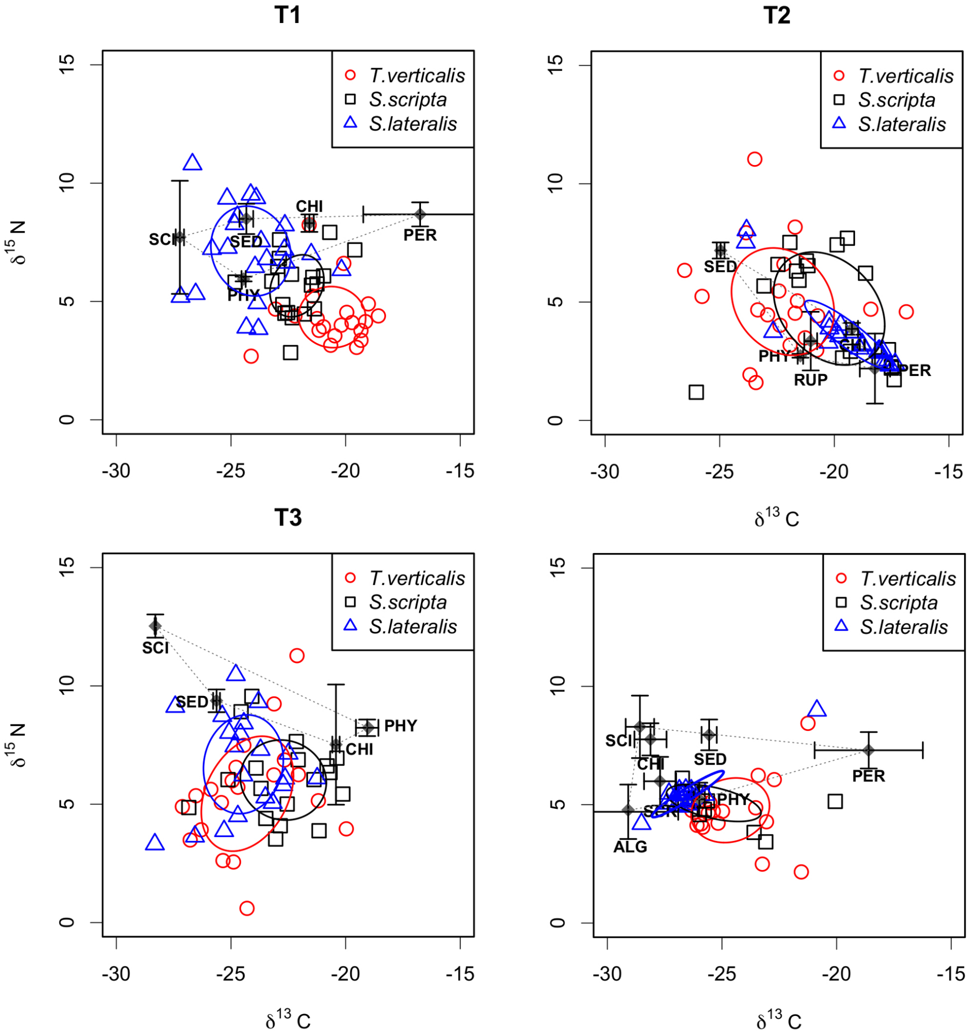

Figure 4. Dual-isotope food web diagram of 4 temporary sites showing the Standard ellipse area (SEAc) of *Trichocorixa verticalis, Sigara lateralis* and *Sigara scripta*. Dashed lines represent the mixing polygon circumscribed by the isotopic signature of several resources (mean δ^{13}C and δ^{15}N±SD). Corixid δ^{13}C and δ^{15}N values were corrected for trophic enrichment. ALG= filamentous algae; CHI= chironomidae; PHY = phytoplankton; SCI= *Scirpus* sp.; PER =periphyton; SED= sediments; RUP= *Ruppia* sp.; SCR= *Scirpus* sp.+*Ruppia* sp.

Table 1 Stable isotope values (mean±SD) of *Trichocorixa verticalis* (TV), *Sigara lateralis* (SL) and *S. scripta* (SS) in temporary waters during summer (June 2011).

Sites	Species	*n*	δ^{13}C±SD	δ^{15}N±SD
T1	TV	20	-20.1 ± 1.4	6.6 ± 1.3
	SL	21	-23.6 ± 1.7	9.4 ± 1.8
	SS	19	-21.6 ± 1.1	7.9 ± 1.3
T2	TV	20	-21.7 ± 2.2	7.3 ± 2.2
	SL	23	-18.9 ± 1.9	5.9 ± 1.4
	SS	18	-19.8 ± 2.3	7.0 ± 2.3
T3	TV	20	-23.8 ± 2.0	7.7 ± 2.4
	SL	20	-23.9 ± 1.8	8.9 ± 2.6
	SS	18	-22.2 ± 1.7	8.3 ± 2.0
T4	TV	19	-24.1 ± 1.7	7.1 ± 1.3
	SL	18	-25.9 ± 1.5	7.7 ± 0.9
	SS	13	-24.9 ± 2.0	7.4 ± 0.7

Local names for these sites are as follows: T1= 9N3PP; T2 =AC3; T3= Entremuros; T4= Lucio del Lobo (see Frisch et al. 2012 for details). See Figure 1 for location.

Estimated trophic position within habitats

The estimated mean trophic position was at 3.3 for *S. lateralis* and at 2.1 for *T. verticalis* in the laboratory experiment (Fig. 2). Similarly, in permanent ponds S. *lateralis* fed at a higher trophic level (3.4) than *T. verticalis* (2.4). In temporary waterbodies the mean estimated trophic position was at 2.1 for S. *lateralis*, at 1.9 for *S. scripta* and at 1.7 for *T. verticalis*.

Niche width differences among species

SEA_B values did not differ significantly between *T. verticalis* and *S. lateralis* within each aquarium where they co-existed, and SEA_c showed complete segregation (Table 2).

SEA_B comparisons between *T. verticalis* and *S. lateralis* were not performed within each permanent pond and between them, as their large isotopic niche widths could be largely the result of spatial variation, preventing a reliable comparison of their dietary and isotopic niche variability. Nevertheless, it was evident that no SEA_c overlap existed between species at A3 and B3.

In temporary waters, *T. verticalis* had a significantly larger SEA_B than *S. lateralis* at T2 and T4 (SEA_c 75 and 80%, respectively) (Table 2). At T1 SEA_B was significantly smaller for *T. verticalis* (SEA_c 43%) than for *S. lateralis*. There were no significant differences in SEA_B for *T.*

verticalis (SEA_c 43%) than for *S. lateralis*. There were no significant differences in SEA_B for *T. verticalis* and *S. scripta* (Table 2). Maximum SEA_c overlap was found at T4 between *T. verticalis* and *S. scripta* (27%) and at T3 between *T. verticalis* and *S. lateralis* (31%). These were the only two cases in which the centroid locations for different corixid species (D) were not statistically different (Table 3; Figs. 2-4).

Table 2. Summary of niche width analyses based on Bayesian tools showing: the Isotopic space (SEAc) inhabited by *Trichocorixa verticalis* (TV), *Sigara lateralis* (SL) and *Sigara scripta* (SS), the Bayesian probability that the isotopic space (SEAc) of one species was smaller than that of the other species, and the proportional SEAc overlap. Sample sizes (*n*) are reported for each species in each site. Asterisks indicate significant differences between SEAc.

Sites	***n***			**SEAc (‰²)**			**Bayesian probability (%)**			**SEAc overlap (%)**		
	TV	SL	SS	TV	SL	SS	TV-SL	TV-SS	SS-SL	TV-SL	TV-SS	SS-SL
AQ1	8	8	/	1.04	0.43	/	32	/	/	0	/	/
AQ2	10	8	/	0.19	0.40	/	52	/	/	0	/	/
T1	20	21	19	5.93	10.34	4.73	96*	25	99*	0	5	4
T2	20	23	18	15.67	3.88	17.36	0*	61	0*	5	23	15
T3	20	20	18	14.54	11.38	9.94	22	12	67	31	24	18
T4	19	18	13	7.26	1.45	4.31	0.09*	10	4*	0.16	27	12

Table 3. P values for comparison of mean centroid position between *Trichocorixa verticalis* (TV), *Sigara lateralis* (SL) and *Sigara scripta* (SS) according to the methodology developed by Turner et al. (2010). Significant differences are indicated by asterisks.

Sites	**Species comparison**		
	TV-SL	TV-SS	SS-SL
AQ1	0.001*	/	/
AQ2	0.001*	/	/
A3	0.001*	/	/
B3	0.001*	/	/
T1	0.001*	0.001*	0.001*
T2	0.001*	0.008*	0.084
T3	0.106	0.018*	0.008*
T4	0.001*	0.297	0.073

Discussion

To our knowledge, this is the first isotopic study that compares trophic relationships between invasive and native aquatic corixids from different ecosystems, supported by an experimental approach. Although we encountered some of the limitations observed in other isotopic studies, including spatial and temporal variability in basal resources, we found evidence of changes in the relative position of $\delta^{13}C$ and $\delta^{15}N$ values between co-existing invasive and native corixids among the studied habitats. Stable isotopes revealed strong resource partitioning between species with little or no isotopic niche overlap in permanent ponds when water levels are relatively stable, but also some degree of niche overlap in unstable temporary sites.

Trophic relationships between *T. verticalis* and native corixids

Permanent ponds and aquaria

A prerequisite for species co-existence in stable habitats, assuming that consumers and resources are all in equilibrium, is that species must exploit different resources to survive (Hutchinson, 1958). We found consistent differences in both $\delta^{13}C$ and $\delta^{15}N$ values between the native and the invasive species in both permanent ponds or experimental aquaria, and no isotopic niche overlap between them. This suggests that *T. verticalis* and *S. lateralis* fed on different food items, facilitating their co-existence, at least in these stable systems.

Omnivorous corixids such as *T. verticalis* and *S. lateralis* (Murillo & Recasens, 1986; Simonis, 2013) possess similar piercing-sucking mouthparts (stylets) suitable for feeding on both plants and animals. However, previous studies have confirmed the importance of animal prey to *S. lateralis* (Murillo & Recasens, 1986; Layer et al., 2010) and others have described *T. verticalis* as predators on Cladocera and *Artemia* in its native range (Wurtsbaugh, 1992; Simonis, 2013). In contrast, our results showed that *T. verticalis* fed at lower trophic level than *S. lateralis* both in the experimental aquaria and in permanent ponds, and suggest a tendency of *T. verticalis* towards herbivory and the higher importance of animal prey for *S. lateralis.* This may partly be explained by the salinity of the permanent ponds we studied, which are too saline for cladocerans but are not hypersaline and have no *Artemia* (Frisch et al., 2006). Our data did not allow a detailed assessment of corixid diets, as many corixids often fell outside the polygons that could be constructed with measured resources within each system, therefore, we are not able to estimate if small or big differences exist in their diet. However, plants and animals from the laboratory experiment had sufficiently distinct $\delta^{13}C$ values to aid approximate identification of their diet. In fact, the close isotopic value of Chironomidae and Oligochaeta to *S. lateralis* (Fig. 2) suggested their inclusion in its diet. In contrast, *T. verticalis* did not appear to be heavily reliant on animal prey (Fig. 2).

There was also evidence of larger inter-individual variation in the field than in the laboratory experiment for both species. Wide isotopic variation in wild populations is common in nature, due to sexual or age differences, diet specialization and spatial variation (Fry et al., 1999; Layman & Allgeier, 2012). While we were unable to pinpoint the exact mechanism behind this variation, it seems possible that spatial heterogeneity in basal resource values, within or among ponds, explains the clustering of isotopic values we observed in each corixid species in permanent ponds, and the much greater isotopic variation observed there for *T. verticalis* and *S. lateralis* than in experimental aquaria.

Isotopic composition of sources can vary at very small scales (< 1 m) within a wetland (Hill et al., 2008). Similarly, our results revealed considerable isotopic variation for sources within a single permanent pond (Fig. 3), and how patchiness can affect the isotopic composition of consumers in aquaria (Fig. 2).

Therefore, it seems possible that, despite their ability to fly between waterbodies, corixids in permanent ponds are very local in their feeding strategy and faithful to a patch, their isotopic values reflecting the patch availability of the resources. However, individuals may also disperse and feed among ponds that differed in basal resource values. Our result did not support this hypothesis, as we did not find spatial differences (among permanent ponds) in the isotopic values of sources. However, these were tested only for a few common sources whose isotopic composition changes very quickly, so we cannot rule out the possibility that we would have found a different result using less variable sources. These two possibilities need to be investigated in the future by more detailed spatial analyses of consumers and sources, as such high patch fidelity would represent a very surprising result for a non-sessile organism.

Temporary waterbodies

During summer, with high temperatures and no rainfall, temporary aquatic habitats in Doñana quickly reduce their depth and surface area and dry out completely by August (Serrano et al., 2006; Frisch et al., 2012). In these conditions, corixid densities become very high and opportunities for *S. lateralis* to feed on preferred prey may decrease, as other invertebrates become scarce. At the same time however, a high quantity of detritus caused by the decomposition of organic matter becomes progressively mixed and concentrated in shallow waters where corixids concentrate. In these conditions of disturbance and superabundant food of low diversity, we might expect convergence of diet between different corixid species. However, isotopic values of different corixid species were very similar at some of the four temporary waterbodies but very different at others. Similar isotopic values do not necessarily indicate similar diet, as corixids can feed on sources that are not distinct enough isotopically to allow discrimination. Similarly, isotope niche widths were site specific for each species (Fig. 4). This large intra and interspecific variation in isotopic composition could indicate the influence of various physiological processes, including life stage, growth rate or body size (Fry & Arnold, 1982; Haubert et al., 2005; Carleton & Martínez del Rio, 2005), but can also indicate a more varied diet (Bolnick et al., 2003) or differences in the magnitude of variation of basal resources among temporary sites (Bearhop et al., 2004).

We are unable to assess the relative contribution of these factors to the observed variability in species isotopic composition. However, irrespective of the mechanisms behind such variation, our data revealed that some degree of niche overlap might also occur between the invasive and native corixids in highly variable systems (Fig. 4). As a consequence, we suggest the possibility that, within these populations of generalist feeders, some individuals of both species exploit similar resources.

Nonetheless, niche overlap is not sufficient to indicate that competition between species occurs in these habitats, especially without data that directly quantifies limitation or superabundance of food. However, the existence of niche partitioning and the use of similar resources among native and invasive corixids indicate that competitive interactions exist between them. Furthermore, *S. lateralis* seemed to occupy a slightly lower position in temporary waters respect to permanent ponds or aquaria. This is not surprising, since native species are probably more adapted to cope with such environmental and food fluctuations. Therefore, it seems possible that higher dietary plasticity of the native species is the mechanism behind its co-existence with *T. verticalis*.

Trophic ecology of *T. verticalis* in stable permanent ponds

Stable isotopes suggest that the invasive species tends to be herbivorous in stable waters. This result agrees with previous studies and personal observations that indicate *T. verticalis* has a herbivorous strategy at the first instar stage (Campbell, 1979; Kelts, 1979), and a diet based on periphyton as adults outdoors (Downing, 2005; authors' personal observation). Periphyton is a mixture of algae, cyanobacteria, heterotrophic microbes, detritus and mucilaginous polysaccharides, and each of these is likely to have very different isotopic values to the whole structure analysed during this study. It is possible that *T. verticalis* assimilated only some components from this mixture. A periphyton-based diet is also supported by close relationships between δ^{13}C values of *T. verticalis* from permanent ponds with those of periphyton during winter (Supplementary Fig. 2).

Contrary to the typical winter ^{15}N-depletion (Harrod & Grey 2006), which was found for POM and epipelon during this study, *T. verticalis* was ^{15}N-enriched. This suggests this seasonal variation in *T. verticalis* δ^{15}N values was not related to change in the importance of periphyton in its diet, but was most likely explained by seasonal changes within periphyton composition and its δ^{13}C and δ^{15}N values (Borduqui, Ferragut & Bicudo, 2008) (Supplementary Fig. 2). Thus, *T. verticalis* could be assigned to the grazer guild in our permanent pond system.

Varying use of periphyton between permanent ponds and aquaria may partly reflect differences in its composition with their different water chemistry (e.g. salinity, see Online Resource 1), or with the substratum where growth occurs (i.e. epilithon, epipelon, epiphyton, periphyton). At higher nutrient concentrations, such as those of the permanent ponds, periphyton may contain a higher proportion of green algae, more palatable to grazers (Rejmánková & Komárková 2005).

Different isotopic values for nymph and adult *T. verticalis* suggest an ontogenetic dietary shift, as found by Simonis (2013). However, different dynamics of tissue turnover between life stages (Haubert et al., 2005) or increases in consumption rate during ontogeny (Simonis 2013), could also contribute to isotopic shifts between nymphs and adults, at least during winter. Further studies (e.g. metabarcoding of gut contents) are needed to clarify this point, as ontogenetic diet shift can play an important role in explaining the success of some organisms in the invaded ecosystem, by allowing a better use of resources and/or reducing competition (Céréghino, 2006).

Conclusion

We showed how trophic interactions between native and invasive corixids changes between permanent and temporary waterbodies. These changes seem to indicate that competition among corixids is not equal between habitats that differed in the types and abundances of local food sources. Although these are results from one sampling campaign, and we cannot exclude the possibility that results would be different in other periods of the year, our findings help to elucidate the processes underlying the co-existence of these species, and suggest many questions about this invasion that need to be addressed in the future.

There is a need for further studies to fully understand the habitat and foraging ecology of *T. verticalis* in its introduced range, especially since the species is expected to spread across a large part of Europe (Guareschi et al., 2013). Indeed, *Trichocorixa* has an important effect on ecosystem functioning in its native range (Downing, 2005), and long-term field and mesocosm studies are required to assess the impact of this invasive species on the native corixid community (including species not covered in the present study), as well as on prey assemblages (e.g. through trophic cascades via predation on zooplankton, Simonis, 2013). The effect of the invader in other habitat types where it is present in Iberian Peninsula, such as solar saltworks (Van de Meutter et al., 2010; authors, unpublished) should also be addressed.

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Supplementary Materials

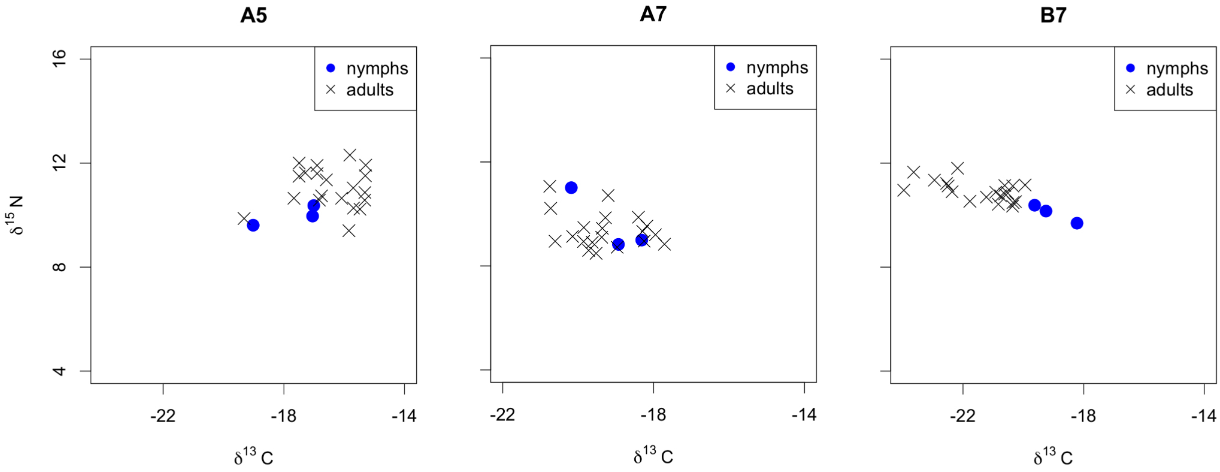

Figure 1. Dual-isotope food web diagram within three permanent ponds (A5, A7 and B7) illustrating isotopic differences in *Trichocorixa verticalis* adults and nymphs during winter.

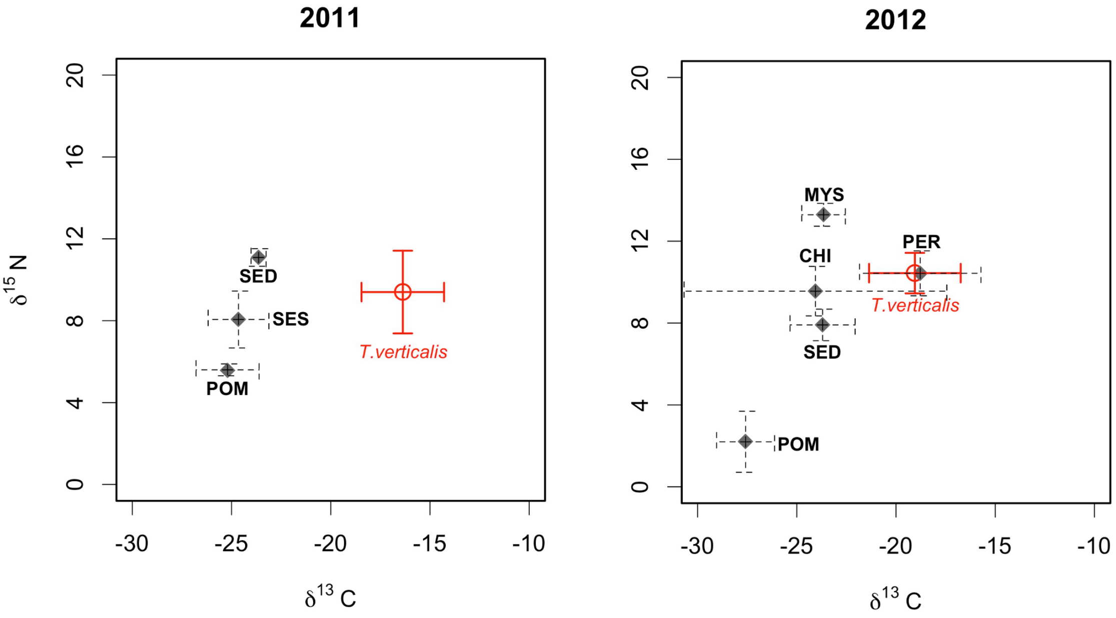

Figure 2. Dual-isotope food web diagram of three permanent ponds (A5, A7 and B7) illustrating isotopic seasonal (left: summer; right: winter) and spatial variation in *Trichocorixa verticalis* and common food web components within seasons: CHI=chironomidae; POM= particulate organic matter, MYS= *Mesopodopsis slabberi*; SED= sediments; SES=seston.

Table 1. Salinity and Chlorophyll a variation in temporary and permanent waterbodies across seasons.

Sites	Season	Chl *a* (μg l^{-1})	Salinity (psu)
T1	summer	36.4	7.6
T2	summer	105.5	15.9
T3	summer	44.6	8.7
T4	summer	76.7	5.4
A3	summer	55.5	9.1
B3	summer	145.5	9.3
A7	summer	155.0	19.8
B7	summer	135	18.6
A5	summer	157.0	8.7
A7	winter	23.7	18.3
B7	winter	53.7	11.6
A5	winter	15.5	10.9

Table 2. Sampling methodology used across temporary and permanent waterbodies.

Species	Sampling methods
Corixids (adults and juveniles)	D-framed pond net (500 μm mesh; 16 × 16 cm)
Benthos	D-framed pond net (500 μm mesh; 16 × 16 cm) Cylindrical ($32cm^2$) cores. Box ($240cm^2$) cores.
Zooplankton	Tows (200 μm and 500 μm sizes). Concentrated in laboratory on a 64 μm mesh
Mysidacea	Fixed traps (Fyke type. funnel-mouthed bags trap) with three mesh sizes: 2 mm, 5 mm and 10 mm.
Periphyton	Submerged part of plants were brushed with a toothbrush, then vigorously shaked in distilled water
Sediments (i.e. epipelon)	Benthic corer (top 5 mm)

Table 3. Standard references used at UC Davis and at LIE laboratory.

	$\delta^{13}C$ (‰VPDB)	**%C**	**$\delta^{15}N$ (‰Air)**	**%N**
UC Davis				
Nylon	-27.81		-9.77	
Bovine Liver	-21.69		7.72	
USGS-41 Glutamic Acid	37.626		47.6	
Glutamic Acid	-28.85	40.81	-4.26	9.52
Peach leaves	-26.12	46.18	1.95	2.88
LIE				
Cow Horn	-22.49 ± 0.12		9.94 ± 0.14	
Whale Baleen	-22.48		9.92	
Feathers of Razorbill	-15.72 ± 0.08		16.55 ± 0.2	

Table 4. Range of stable isotope values of source items (n=3) in 4 temporary waters: PER= periphyton; PHY= phytoplankton; SCI= *Scirpus* sp.; RUP = *Ruppia* sp.; SCR= *Scirpus sp.* root; ALG= filamentous algae; CHI= chironomids; SED = sediments.

Sites	**Source**	**Range**	
		$\delta^{13}C$	**$\delta^{15}N$**
T1	PER	-19.1 - 14.1	8.1 - 9.0
	PHY	-24.5 - 24.3	5.8 - 6.0
	SCI	-27.4 - 27.0	5.0 - 9.7
	CHI	-21.8 - 21.4	7.9 - 8.7
	SED	-24.7 - 24.1	7.8 - 9.0
T2	PER	-18.6 - 17.5	1.2 - 3.9
	PHY	-21.6 - 21.4	2.7 - 2.8
	RUP	-21.3 - 20.7	2.3 - 4.7
	CHI	-19.5 - 19.1	3.7 - 4.2
	SED	-25.1 - 24.8	6.9 - 7.5
T3	PHY	-19.4 - 18.5	7.9 - 8.6
	SCI	-28.3 - 28.2	12.0 - 13.0
	CHI	-20.5 - 20.2	4.6 - 9.0
	SED	-25.8 - 25.5	9.5 - 9.8
T4	PER	-21.0 - 16.3	6.7 - 8.1
	PHY	-26.1 - 25.7	4.7 - 6.0
	ALG	-31.3 - 26.9	3.9 - 6.1
	SCI	-29.3 - 28.1	7.3 - 9.8
	SCR	-28.5 - 27.1	4.9 - 6.9
	CHI	-28.6 - 27.3	7.2 - 8.5
	SED	-25.9 - 25.2	7.6 - 8.7

Chapter 3

Parasitism by water mites in native and exotic Corixidae. Are mites limiting the invasion of the water boatman *Trichocorixa verticalis* (Fieber, 1851)?

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Abstract

The water boatman *Trichocorixa verticalis verticalis* (Fieber 1851) is originally from North America and has been introduced into the southern Iberian Peninsula, where it has become the dominant Corixidae species in saline wetlands. The reasons for its success in saline habitats, and low abundance in low salinity habitats, are poorly known. Here we explore the potential role of water mites, which are typical parasites of hemipterans, in the invasion dynamics of *T. verticalis*. We compared infection levels between *T. verticalis* and the natives *Sigara lateralis* (Leach, 1817) and *S. scripta* (Rambur, 1840). No mites were found in saline wetlands where *T. verticalis* is highly dominant. Larvae of two mite species were identified infecting corixids in habitats of lower salinity: *Hydrachna skorikowi* and *Eylais infundibulifera*. Total parasite prevalence and prevalence of *E. infundibulifera* were significantly higher in *T. verticalis* compared with *S. lateralis* and *S. scripta*. Mean abundance of total infection and of *E. infundibulifera* and *H. skorikowi* were also higher in *T. verticalis*. When infected with *H. skorikowi*, native species harbored only one or two parasite individuals, while the smaller *T. verticalis* carried up to 7 mites. When infected with *E. infundibulifera*, native species harboured only one parasite individual, while *T. verticalis* carried up to 6. Mite size didn’t differ among host species, suggesting that all are suitable for engorgement. Both mite species showed a negative correlation between prevalence and salinity. *T. verticalis* susceptibility to parasitic mites may explain its low abundance in low salinity habitats, and may contribute to the conservation of native corixids. The success of *T. verticalis* in saline wetlands may be partly explained by the absence of parasitic mites, which are less halotolerant.

Introduction

Invasive species have become a major conservation problem in aquatic ecosystems at the global scale (Leppäkoski et al., 2002). Understanding the interactions between invasive species and the recipient community (including free living organisms and parasites) is key to understanding the invasion process, to improve our capacity to predict the outcome of invasions and to design strategies for the conservation of native taxa.

The introduction and spread of invasive species is a significant but insufficiently studied factor in disease emergence (Kelly et al., 2009a; Mastitsky et al., 2010). Although it is now widely recognized that the impacts of species introductions on native communities are often mediated via parasites (Prenter et al., 2004, Dunn, 2009), our understanding of how such impacts occur is incomplete. Most studies have focused on the effect of the loss of coevolved parasites during the introduction process ('Enemy Release Hypothesis', Torchin et al., 2002, 2003; Keane & Crawley 2002; Colautti et al., 2004; Prenter et al., 2004), and the introduction of exotic parasites arriving with alien hosts to the recipient community ('Parasite Spillover', Dobson & Foufopoulos 2001; Power & Mitchell 2004). However, with the exception of native parasites affecting exotic plants and invertebrates of economic importance, which have been the subject of studies of biological control (Williams et al., 2003; Li et al., 2012), the acquisition of new parasites by exotic species has been largely overlooked, even though it is potentially a frequent and important process (Kelly et al., 2009b; Mastitsky et al., 2010). Depending on the mechanism and the role played by the novel parasite, the consequences for the invasion success of the alien host and the impact on the recipient community can be highly variable. Disentangling such mechanisms will improve our understanding of biological invasions and enhance our ability to predict the outcomes of ongoing and future invasions.

Trichocorixa verticalis verticalis (Hemiptera: Corixidae) is native to North America and occurs in brackish and saline wetlands (Sailer, 1948). Recently it has invaded aquatic ecosystems in Africa, Oceania and Europe, where it is the only known exotic corixid (Rabitsch, 2008, 2010; Guareschi et al., 2013). It is predicted to spread extensively across Europe during the course of this century (Guareschi et al., 2013). However, there are currently few data on its potential ecological impact in the introduced range. In its native North America, this omnivorous insect is important in structuring the pelagic planktonic communities of aquatic ecosystems through predation on cladocerans (Simonis, 2013) and anostracans (Wurtsbaugh, 1992). In Great Salt Lake (USA), during periods of low salinity, *T. verticalis* has been shown to affect the food web of the lake through its predation on brine shrimp *Artemia franciscana* Kellog, 1906 (Wurtsbaugh, 1992). It causes a strong trophic cascade affecting microbes and phytoplankton (Wurtsbaugh, 1992). Therefore, we can expect *T. verticalis* to have a significant impact in wetlands of the introduced range.

In its introduced range in the south of the Iberian Peninsula, *T. verticalis* is highly dominant and abundant in permanent saline fish ponds and salt ponds where native Corixidae are rare and may have been competitively excluded. In contrast, native corixids dominate in seasonal ponds and marshes of lower salinity within the same general area (Rodríguez-Pérez et al., 2009; Van de Meutter, Trekels & Green, 2010). This strong pattern in relation to salinity remains unexplained, especially as experiments with adult corixids have shown that *T. verticalis* adults perform well at low salinities and are not more resistant to high salinities than some native corixids (Van de Meutter et al., 2010; Coccia et al., 2013). Indeed, the native *Sigara selecta* (Fieber, 1848) is more halotolerant than *T. verticalis* (Van de Meutter et al., 2010).

There is no previous information on the potential role of parasites in the invasion dynamics of the American corixid. Corixidae are known to be hosts to a diverse community of parasites, water mites (Hydracarina) being among the most common (Reilly & McCarthy 1991). Parasitic mites occur in almost all fresh and brackish aquatic environments, where they can reach densities of more than 2000 specimens per square meter (Smith et al., 2010). While most nymphal and adult stages are predatory and free living in aquatic ecosystems, the larval stage is parasitic (Davids, 1973). Mites can strongly impact host populations and influence biological interactions between corixid species (Smith, 1977). Therefore, they have the potential to play an important role in the outcome of competition between native and invasive species. However, there are no previous studies of parasitic mites in the Corixidae of the southern Iberian Peninsula. On the other hand, there exists a lack of information about factors affecting host preference by water mites. Size of hosts appears to be one important factor (Blower & Roughgarden 1988) and the difference in size between *T. verticalis* and native species may potentially influence parasitism rates and hence the success of the invasion.

The aim of this study was to compare infestation levels of larval water mites in native (*Sigara lateralis* and *Sigara scripta*) and exotic corixids (*T. verticalis*) along the salinity gradient in Doñana in southwest Spain, and to consider their role in the invasion of *T. verticalis*. We test the following hypotheses: (i) *T. verticalis* is released from mite parasitism at the high salinities where it dominates; (ii) parasites grow to a larger size in larger corixid species; and (iii) mites attach to a wider range of body parts in *T. verticalis* because this species is less sclerotized than native species. The results of this study may have important implications for the conservation of native corixid fauna in Europe.

Material & Methods

Study area

The climate in the study area is Mediterranean subhumid, characterized by hot, dry summers and mild winters. Sampling of seasonal habitats where native and invasive corixids coexist was mainly conducted within Caracoles estate in the northern edge of Doñana National Park (Southwest Spain, see Fig. 1). This is a marshland area containing 96 experimental temporary ponds of different size and depth (see Frisch et al., 2012; Sebastián-González & Green, 2014 for details). Experimental ponds are fed mainly by precipitation that occurs generally from late September to early April.

Sampling was also carried out in the Veta la Palma fish ponds (Fig. 1) where *T. verticalis* is the dominant corixid (Rodríguez-Pérez et al., 2009; Rodríguez-Pérez & Green, 2012). Veta la Palma is an extensive fish farm composed of 37 shallow brackish ponds within Doñana Natural Park. These permanent, saline, ponds are supplied with water from the estuary of the River Guadalquivir (see Kloskowski et al., 2009; Rodríguez-Pérez & Green, 2012 for details). In general, the fish ponds are much more saline on average than the seasonal marsh and temporary ponds in Doñana (Rodríguez-Pérez et al., 2009; Kloskowski et al., 2009; Van de Meutter, Trekels & Green, 2010).

Details of the sampling sites, dates and sampling objectives are summarized in Table 1.

Specific sampling

On 27 June 2011 a total of 307 adult corixids (111 *T. verticalis*, 103 *S. lateralis* and 93 *S. scripta*) were collected specifically for the study of parasites using a D-framed pond net (500 µm mesh; 16 × 16 cm) from an individual temporary pond in Caracoles estate (hereafter AC3). The sampling date was selected because corixids reached maximum abundance in summer, and this particular pond was chosen based on previous observations of the species coexistence [authors' personal observation]. After collection, individuals were placed inside plastic containers filled with damp aquatic vegetation and transported alive to the laboratory. Once at the laboratory, specimens were carefully separated and individually stored in 1.5 ml Eppendorf tubes filled with 70% ethanol, until examination for parasites. This sampling was designed to minimize the probability of water mites becoming detached from the host prior to examination, in order to have an exact measure of infection rates.

General sampling in Caracoles and Veta la Palma ponds

We also studied the prevalence of infected corixids in a large collection of samples collected from 32 ponds within Caracoles estate, which were representative of all size and depth classes and 10 points within 7 natural or semi-natural waterbodies in the immediate surroundings (Fig. 1) during May-June of two years (2010-2011), as part of a broader study on the invasion of *T. verticalis.* Water salinities vary spatially and temporally, with a range of 2.62 to 37.8 ppt during the study period in the selected sites.

Details of mite infections are presented here for those sites that held both parasitic mites and at least two species of corixids. To establish the prevalence and abundance of mites in the fish ponds where *T. verticalis* is highly dominant, we examined 909 *T. verticalis* adults collected from 3 permanent ponds (G3, A3 and A7, which were representative of the salinity gradient within the pond complex) during May-July 2011 (Fig.1). Water salinities varied from 4.3 to 25.8 ppt during the study period in the selected ponds. These samples were collected in a sweep net as before, but individuals from the same pond were stored together in 5 ml vials filled with 70% ethanol until they were examined for the presence of parasites. Therefore we cannot exclude the possibility of some mites becoming detached from their hosts (although the attachment sites remain visible, see Results).

Some free living adult mites were found in 4 different sites during the general sampling: two temporary ponds within Caracoles estate (AC4 and AE5); one semi-natural pond (FAO pond) within Doñana National Park; and one intermittent stream (Caño Guadiamar) within Doñana Natural Park (during March 2010, and March and May 2011). These samples were used to compare species composition with parasitic larvae and to aid larval identification. Salinity (ppt) was measured in situ using a WTW 340i multiprobe.

Using a stereomicroscope we identified each corixid species in our samples (after Jansson, 1986; Nieser et al., 1994; L'Mohdi et al., 2010), determined its sex and checked for the presence of mites. Body length of corixids were measured on images taken with a digital camera (AxioCam Icc1) connected to a Zeiss microscope (Discovery V8). For the inspection of the thoracic and abdominal torsum, hemielytra and wings were lifted. We measured prevalence (proportion of individuals infected), mean abundance (number of parasites averaged for each corixid species), and mean intensity (number of parasites averaged for all infected corixids) for total mite infection and for each mite species in the different hosts (see Bush et al., 1997 for definitions of infection descriptors).

We recorded the attachment site for each individual mite and compared the susceptible surface area between different hosts using all infected individuals (from both specific and general samplings). Site of attachment was subdivided into different regions: head, pronotum, legs (pairs 1-3 / right-left / femur, tibia, tarsum), abdomen (1-7 abdominal segments) and thorax. All water mites were measured on images, in the same manner as corixids, as indicators of parasite growth (Davids 1973).

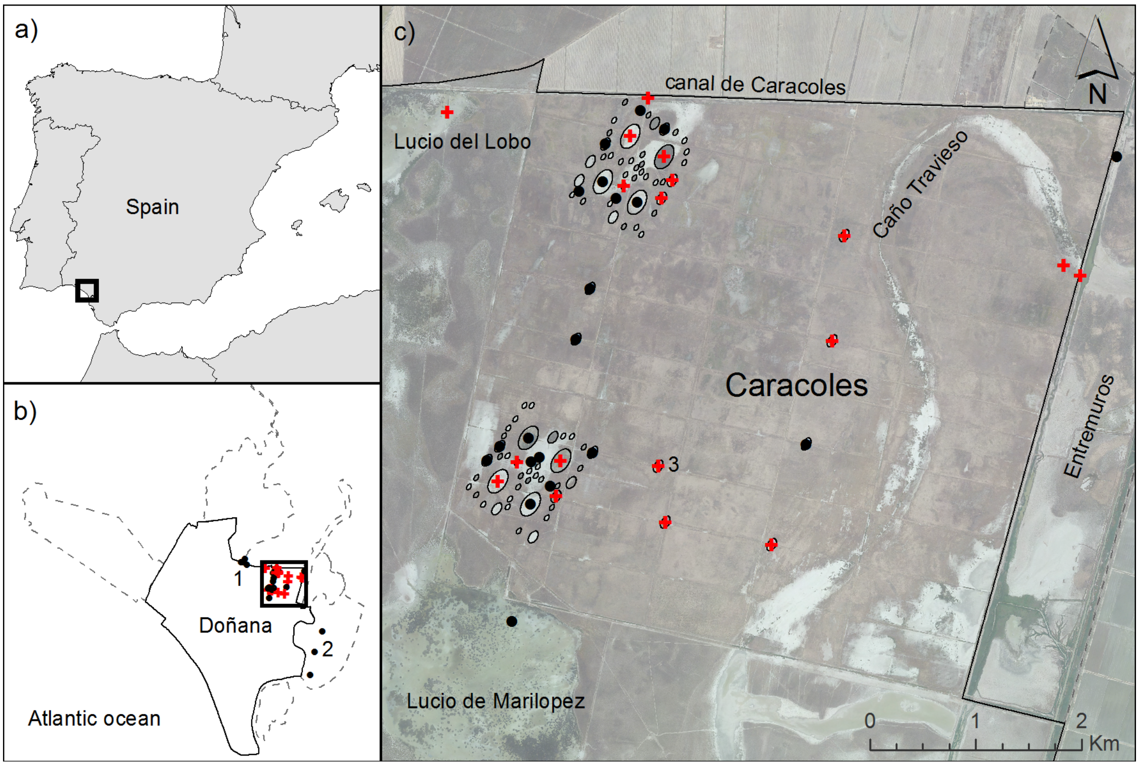

Figure 1. Map of the study area showing the Doñana region in southern Spain (a). The solid lines indicate the boundary of Doñana National Park and the dashed lines indicate Doñana Natural Park (b). The area where we found water mites within Caracoles estate and immediate surroundings is framed. Red crosses represent sites with water mites and corixids; black dots indicate sampled sites without water mites but with corixids. The number (1) indicates FAO sample sites and (2) indicates Veta la Palma fish ponds. Map detail (c) shows the spatial arrangement of all sites with water mites. The number (3) indicates the pond (AC3) of the specific sampling. See Frisch et al. (2012) for further details of the Caracoles ponds.

Table 1. Summary of the location, dates and objectives of sampling.

Sampling Type	General sampling
Main objective	Broader study of seasonal dynamics of corixid communities; plus water mite infections
Study area / total nº ponds / date	Caracoles estate and waterbodies in the immediate surroundings/ 42 ponds / May-June 2010-2012
	Veta la Palma Fish Ponds / 3 ponds (Gaveta 3, A3, A7) / May-July 2011
Sampling Type	Specific sampling
Main objective	Most accurate possible calculation of mite infection rates
Study area / nº ponds / date	Caracoles estate / 1 pond (AC3) / 27 June 2011

Larvae identification

Larvae were inspected under a Zeiss Standard bright-field microscope and a representative subset were detached, slide mounted and studied with a Leica TCS SPE Confocal Laser Scanning Microscope (see Lorenzo-Carballa et al. 2011 for detailed procedure). Serial sections were acquired and subsequently worked out with Fiji/Imagej (ver 1.48d; downloaded from http://fiji.sc/Fiji), Amira (ver 5.5.0) and Photoshop CS5 extended. Morphological diagnostic characters were used to identify *Hydrachna* (Davids, 1973) and *Eylais* (Nielsen & Davids, 1975).

Statistical analysis

We evaluated the significance of the differences between corixid species in prevalence with Z tests (Snedecor & Cochran, 1989) and in abundance and intensity with Kruskal-Wallis and Mann–Whitney U tests. We also used Z tests to compare the prevalence between males and females of each corixid species. The size of the different host was compared with Kruskal Wallis tests followed by pairwise multiple comparisons, and the size of water mite larvae was compared with a Mann-Whitney U-test. The effect of host species, number of parasites per host and salinity on mite size was analyzed using Generalized Linear Models. For this particular analysis we used only data from 2011 because of the low number of infected individuals recorded in 2010.

Generalized linear models with binomial responses were used to test the effect of salinity and sampling date (categorized by months and years) on the presence of water mite larvae. Generalized Linear Models were bias corrected according to Firth (1993). *P* values were always adjusted for multiple comparisons through false discovery rate (Benjamini and Hochberg 1995). Statistical analyses were conducted using Statistica 12.0 (StatSoft, Inc.) and R (v 2.15.3, R Development Core Team 2008).

Results

Larvae of two water mite species infecting Corixidae (Hemiptera: Heteroptera) were identified from Caracoles estate: *Hydrachna skorikowi* Piersig, 1900 and *Eylais infundibulifera* Koenike, 1897 (Acari: Hydrachnellae) (Figs. 2-5). In addition to these two species, *Piona nodata* (Müller, 1776) (Acari: Hydrachnellae) was identified in the sample of adult mites. This species has previously been reported to have populations with females laying small eggs resulting in parasitic larvae and populations producing large eggs resulting in non-feeding larvae (Smith 1988); seasonal shifts in the lifestyle have been also observed, with a parasitic phase produced in winter and a free living one in summer (Böttger, 1962). *P. nodata* can infect other insect groups such as chironomids (Peyrusse et al., 2004).

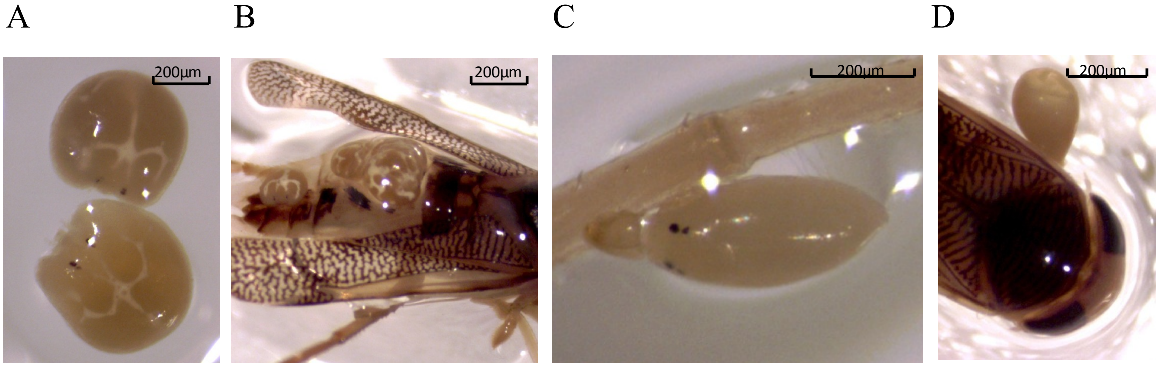

Figure 2. Individuals of *Eylais infundibulifera* (A, B) and *Hydrachna skorikowi* (C, D). These individuals are discoloured by preservation in alcohol. The natural colour of the mites is red due to the presence of carotenoids.

Description of the larvae

Full descriptions of the larvae of *H. skorikowi* and *E. infundibulifera* may be found in Davids (1973) and Nielsen and Davids (1975), respectively. Our identification of specimens agrees with the general descriptions of the larvae and their diagnostic characters. A median margin of the first coxa longer than the lateral margin and a pair of strong setae in the third coxal group are characteristic of the larvae of *H. skorikowi* (Fig. 4). The larvae and protonymph of *E. infundibulifera* has a dorsal plate with converging posterior ridges and a pair of long anterior setae (Fig. 3).

Figure 3. Protonymph of *Eylais infundibulifera* Koenike, 1897. A: Dorsal view. Arrow points to the dorsal plate. Maximum intensity projection. B: ventral view. Arrows point to vestiges of three pairs of legs. Maximum intensity projection. C: Dorsal plate, 3D reconstruction (Amira). Arrow points to diagnostic groove of *E. infundibulifera*.

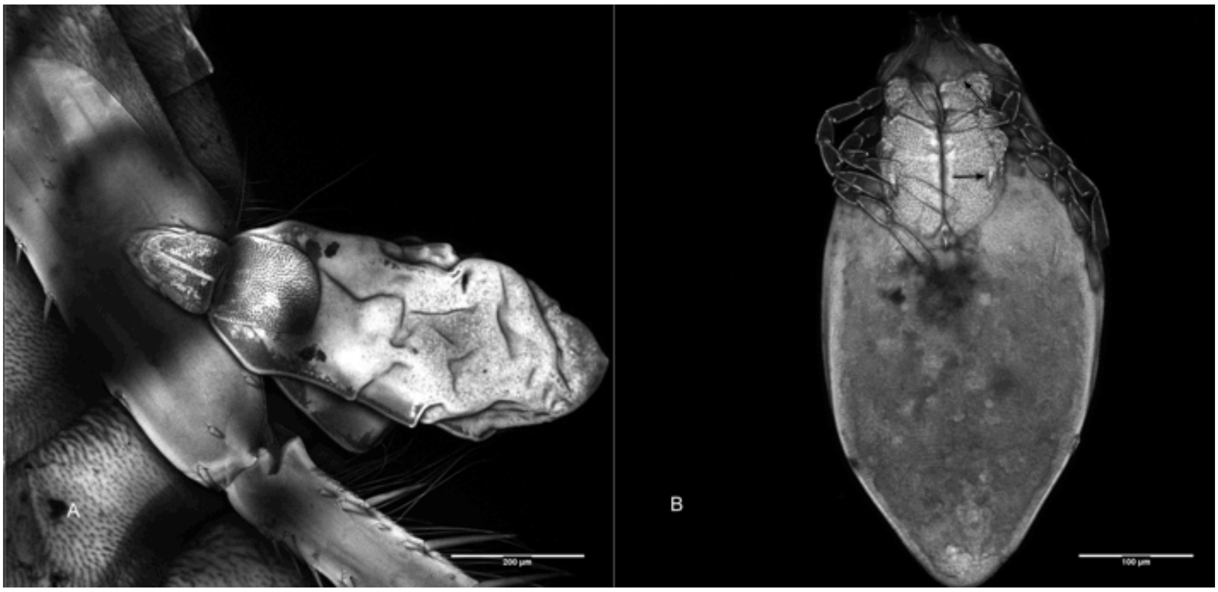

Figure 4. *Hydrachna skorikowi* Piersig, 1900 A: Larva attached to the femur of *Trichocorixa verticalis*. Maximum intensity projection. B: Idiosome, ventral view. The arrow points to a diagnostic character of this species. Maximum intensity projection.

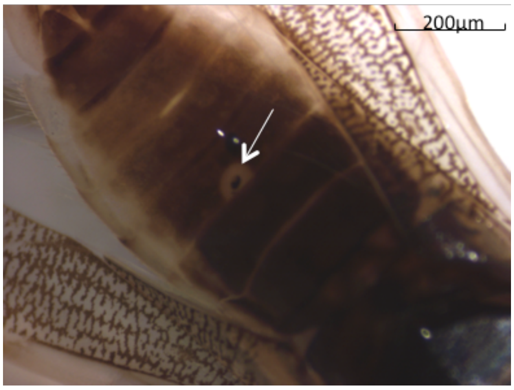

Figure 5. *Sigara lateralis* showing a *brownish spot* which indicates the previous presence of a larval mite.

Infection indexes from the specific sampling in a temporary pond

Total prevalence of water mites at pond AC3 on 27/06/2011 differed among corixid species. *T. verticalis* exhibited the highest values, followed by *Sigara lateralis* and *S. scripta* (Table 2). While the exotic *T. verticalis* was infected by both mite species in this sampling, native corixid species were infected by only one species (*E. infundibulifera* for *S. lateralis* and *H. skorikowi* for *S. scripta*). Paired comparisons (Z tests) showed that differences in total prevalence were significantly higher in *T. verticalis* compared with both *S. lateralis* (Z = 2.705, $P < 0.05$) and *S. scripta* (Z = 2.875, $P < 0.05$). Prevalence of *E. infundibulifera* was significantly higher in *T. verticalis* compared with *S. scripta* (Z = 2.643, $P < 0.05$) but not compared with *S.*

lateralis. Differences in prevalence of *H. skorikowi* among corixid species were not significant. Males and females of the different hosts did not differ in prevalence in total or for either mite species ($P > 0.05$).

Total mean abundance of mites was significantly different between corixid species (Table 2). Pairwise comparisons (Mann Whitney U test) showed that abundance was significantly higher in *T. verticalis* than *S. lateralis* (U = 5100, 5, $P < 0.005$) or *S. scripta* (U = 4563, $P < 0.005$), but did not differ between *S. lateralis* and *S. scripta* (U = 4748, P = 0.63). Mean abundance of *E. infundibulifera* was also significantly different between corixid species (Table 1), being significantly higher in *T. verticalis* than *S. lateralis* (U = 5308, $P < 0.05$) or *S. scripta* (U = 4696, $P < 0.005$), but not differing between *S. scripta* and *S. lateralis* (U = 4696, $P = 0.18$). Mean abundance of *H. skorikowi* was also significantly different between host species (Table 2). Abundance was significantly higher in *T. verticalis* than *S. lateralis* (U = 5407.5, $P < 0.05$), but there were no differences between *T. verticalis* and *S. scripta* (U = 4937, $P = 0.09$) or *S. lateralis* and *S. scripta* (U = 4738, $P = 0.297$).

Total mean intensity of mites was not significantly different between corixid species, neither were there significant differences for either mite species (Table 2, but note the small sample size for native corixids). When infected, native species harbored only one parasite individual; however water mite loads in *T. verticalis* ranged between 1 and 7 parasites per host (1-6 for *E. infundibulifera* and 1-3 for *H. skorikowi*).

Table 2. Prevalence (P%), Mean Abundance (MA ± SE) and Mean Intensity (MI ± SE) of *Hydrachna* and *Eylais* water mite larvae infecting Corixidae from Caracoles estate (pond AC3, Doñana National Park) on 27/06/2011. Compared with Kruskal-Wallis and Mann-Whitney U tests. *P < 0.05, **P < 0.005, ***P < 0.0005. SL= *Sigara lateralis*, SS = *Sigara scripta*, TV = *Trichocorixa verticalis*. H=Kruskal-Wallis H statistic; U= Mann-Whitney U statistic.

	SL	***SS***	***TV***	**H, U**
	(n = 103)	(n = 93)	(n = 111)	
Prevalence (%)				
Eylais	1.94	0.00	9.01	
Hydrachna	0.00	1.07	5.40	
Total	1.94	1.07	12.61	
Mean Abundance (MA ± SE)				
Eylais	0.02 ± 0.014	0.00 ± 0.00	0.22 ± 0.084	H = 12.58**
Hydrachna	0.00 ± 0.000	0.01 ± 0.011	0.08 ± 0.036	H = 7.87*
Total	0.02 ± 0.014	0.01 ± 0.011	0.297 ± 0.101	H = 16.89***
Mean Intensity (MI ± SE)				
Eylais	1.00 ± 0.00	0.00 ± 0.00	2.40 ± 0.618	U = 6
Hydrachna	0.00 ± 0.00	1.00 ± 0.00	1.50 ± 0.342	-
Total	1.00 ± 0.00	1.00 ± 0.00	2.36 ± 0.561	U = 1.76

Infection index from the general sampling in temporary ponds and permanent Veta la Palma fish ponds

Samples collected from fish ponds during May (*n* = 305), June (*n* = 94) and July 2011 (*n* = 510) revealed no evidence of mite parasitism in adult *T. verticalis* (Supplementary Table 1).

From samples collected in May-June 2010-2011 in temporary ponds, we selected the 19 sampling events out of 123 (including ponds in Caracoles estate and natural water bodies in the surrounding area) in which mite parasites and at least two corixid species were recorded. We found similar patterns of parasite infection as for AC3. *S. lateralis* was infected in 9 out of 18 samplings where this species was present (88.8% of infected individuals with *H. skorikowi* and 20% with *E. infundibulifera*); *S. scripta* was infected in 3 out of 13 samplings where it was present (100% of individuals with *H. skorikowi* and 0% with *E. infundibulifera*); *T. verticalis* was found to be infected in 13 out of 17 samplings where it was present (15.4% of individuals with *H. skorikowi* and 92.3% with *E. infundibulifera*) (Table 3). Considering all the samplings (n = 19), *T. verticalis* showed highest values of total prevalence in 13 cases, and *S. lateralis* in 5 cases. The maximum values of prevalence for *H. skorikowi* were 10% for *S. lateralis*, 40% for *S. scripta* and 69.2% for *T. verticalis*; the maximum values for *E. infundibulifera* were 1.47% for *S. lateralis* and 100% for *T. verticalis* (Table 3).

Parasite intensity across the period was 1-2 for *E. infundibulifera* and 1-7 for *H. skorikowi*. When infected with *E. infundibulifera*, *T. verticalis* was infected with 1-2 individuals, while *H. skorikowi* load reached up to 7 individuals per host; native species were infected with only one *E. infundibulifera* per corixid, and only three *S. lateralis* were infected with more than one *H. skorikowi* (2 individuals per host) (Table 3). In addition to *T. verticalis*, *S. lateralis* and *S. scripta*, two other infected corixid species were recorded. *Corixa affinis* Leach, 1817 which was infected in two samplings with *H. skorikowi* (prevalences of 7.14% (n = 14) and 1.44% (n = 69)) and *Sigara stagnalis* (Leach, 1817) which was infected in only one sampling with *H. skorikowi* but with 100% (n = 1) prevalence.

Table 3. Prevalence (%) of *Hydrachna skorikowi* (HS) and *Eylais infundibulifera* (EI) water mite larvae infecting Corixidae (SL= *Sigara lateralis*; SS = *Sigara scripta*; TV= *Trichocorixa verticalis)* from temporary ponds in Doñana National Park. Only samples (ponds/date) where parasites and at least two corixid species were present are included here (37.8% of the total number of samples). Data from AC3, 27/06/2011 (Table 2) are not included.

				SL			*SS*			*TV*	
Pond	**Date**	**Salinity**	***n***	**HS**	**EI**	***n***	**HS**	**EI**	***n***	**HS**	**EI**
ENTREMUROS 1	11/06/2010	2.7	35	2.86	0	0			3		0
LUCIO DEL LOBO	11/06/2010	2.2	54	3.7	0	20	0	0	14	0	7.14
0N1GP	14/05/2011	2.3	32	6.25	0	5	0	0	1	0	0
6N2MP	14/05/2011	4	10	10	0	5	0	0	0		
AC3	15/05/2011	1.2	129	8.53	0	5	40	0	13	69.23	0
AC4	15/05/2011	1.3	25	16	0	0			0		
AE6	15/05/2011	1.5	51	3.92	0	0			2	0	0
AE8	15/05/2011	3.4	57	3.51	0	4	0	0	1	0	0
0N2GP	24/06/2011	4.4	9	0	0	4	0	0	1	0	100
3N3MP	24/06/2011	8.5	1	0	0	0			1	0	100
6N2MP	24/06/2011	20.9	2	0	0	0			10	0	20
9N3PP	24/06/2011	7.6	241	0	1.24	38	2.63	0	26	0	15.38
0S2GP	25/06/2011	8.7	46	0	0	0			30	0	10
0S4GS	23/06/2011	23.7	21	0	0	38	0	0	89	0	3.37
3S3MP	23/06/2011	21	31	0	0	7	0	0	34	0	2.94
5S1PP	23/06/2011	5.9	136	0.74	1.47	1	0	0	10	0	20
AC3	25/06/2011	15.8	43	0	0	30	0	0	53	1.89	5.66
AC4	25/06/2011	23.9	72	0	0	35	0	0	71	0	1.41
CANAL CARACOLES	26/06/2011	8.4	4	0	0	21	4.76	0	16	0	18.75

Determinants of water mite prevalence

Generalized Linear Models indicated that water salinity was a significant predictor for the occurrence of both *E. infundibulifera* ($P < 0.001$) and *H. skorikowi* ($P = 0.018$) Table 4). In both cases, there was a negative partial effect, such that prevalence was lower at higher salinities when controlling for date and corixid species. The prevalence of *E. infundibulifera* was significantly lower in either of the two native corixid species than in *T. verticalis*. Similarly, the prevalence of *H. skorikowi* was significantly lower in *S. lateralis* than in *T. verticalis*. Prevalence was lower in *S. scripta* than in *T. verticalis*, but not significantly so (Table 4). Sampling date also significantly affected mite presence. The occurrence of *E. infundibulifera* was significantly higher in June 2011 than in June 2010 or in May 2011. For *H. skorikowi,* its presence was significantly higher in May 2011 than in May or June 2010, and significantly higher in June 2011 than in June 2010 (Table 4).

Table 4. Results from a GLM with binomial error estimating *Eylais* or *Hydrachna* presence according to sample date, salinity and corixid species (SL = *Sigara lateralis*; SS = *Sigara scripta;*). *Trichocorixa verticalis* (TV) was used as the reference category for the presence of water mite larvae infecting native Corixids (i.e. TV was aliased), as no significant differences in the prevalence of mites were found between native species. Reference groups for sampling date are those on the right. Asterisks indicate statistically significant predictors.

Coefficients	Estimate	Std. Error	Pr(>|z|)	Odds ratio
EYLAIS				
Salinity	-0.116	0.031	< 0.001 *	8.873e-01
SL	-2.910	0.480	< 0.001 *	5.036e-02
SS	-3.553	1.399	0.011*	6.931e-09
05/10 vs 06/10	-1.217	1.608	0.449	1.469e+06
05/10 vs 05/11	-1.119	1.996	0.574	8.462e-01
05/10 vs 06/11	2.097	1.466	0.153	6.010e+07
06/10 vs 05/11	0.097	1.621	0.952	1.102
06/10 vs 06/11	3.313	0.866	< 0.001 *	27.486
05/11 vs 06/11	3.216	1.450	0.026 *	24.944
HYDRACHNA				
Salinity	-0.284	0.146	0.018 *	0.752
SL	-1.868	0.419	< 0.001 *	0.154
SS	-0.618	0.609	0.310	0.539
05/10 vs 06/10	0.250	1.512	0.868	1.284
05/10 vs 05/11	3.341	1.418	0.018 *	28.259
05/10 vs 06/11	2.165	1.600	0.176	8.717
06/10 vs 05/11	3.091	0.601	< 0.001 *	22.006
06/10 vs 06/11	1.915	0.815	0.019 *	6.787
05/11 vs 06/11	-1.176	0.819	0.151	0.308

Relation between host size and mite size

We found differences in body length of hosts among corixid species (Kruskal Wallis test, H = 128.83, $P < 0.001$), *S. lateralis* being the biggest (4.73 ± 0.25 mm) followed by *S. scripta* (4.39 ± 0.25 mm) and *T. verticalis* (4.16 ± 0.32 mm). All pairwise comparisons were statistically significant ($P < 0.05$). The two water mite larvae species didn't differ in size (mean ± SE: 534.98 ± 23.18 µm for *E. infundibulifera* and 535,29 ± 24.40 µm for *H. skorikowi*: U = 575.5, $P = 0.985$). On the other hand, the size of *E. infundibulifera* was very similar between host species (mean ± SE: 573.69 ± 22.10 µm for *S. lateralis* and 566.52 ± 13.92 µm for *T. verticalis*). The same was true for *H. skorikowi* (549.40 ± 62.57 µm for *T. verticalis*; 506.88 ± 46.81 µm for *S. lateralis*; 617.68 ± 207.37 µm for *S. scripta*). Accordingly, the results of a Generalized Linear Model of mite size indicated no significant effect of host species, salinity, nor the number of parasites infecting the host ($P > 0.194$ for *H. skorikowi* and $P > 0.181$ for *E. infundibulifera*). Nonetheless, the date of sampling (May or June) significantly affected the size of *H. skorikowi* ($F_{1, 38} = 25.498$, $P = 0.00001$) with bigger larvae in June. For *E. infundibulifera*, we didn't include the effect of date in the Generalized Linear Model because this mite species was only present in June.

Differences in attachment sites between mite and host species

Attachment sites were highly specific for both mite species. *E. infundibulifera* invariably attached to the dorsal side of the abdomen (Figure 2, a-b); *H. skorikowi* (Figure 2, c-d) mainly selected the legs but it was also found on the hemelytra, abdomen, head and pronotum (Table 5). *E. infundibulifera* was found attached over a higher surface area when infecting *T. verticalis* (2-5 abdominal segments) compared to *S. lateralis* (2-3 abdominal segments) (Table 5). *H. skorikowi* attached over a higher diversity of sites when infecting *T. verticalis* (legs, abdomen, head, and pronotum, in order of declining frequency) followed by *S. lateralis* (legs, abdomen and hemelytra) and *S. scripta* (legs and head) (Table 5). When attached to the legs there was no significant difference between the proportions on the right and left sides.

Table 5. Attachment sites of *H. skorikowi* and *E. infundibulifera* when infecting *Sigara lateralis* (SL), *Sigara scripta* (SS) and *Trichocorixa verticalis* (TV) from Doñana. Data correspond to both the "specific sampling" and "general sampling" (Table 1).

Corixid species	Water mite species	Area of attachment (%)	Specific point (%)	
SL	*E. infundibulifera*	abdomen (100)	segment II (20)	
			segment III (80)	
	H. skorikowi	legs (92.8)	leg I (7.7)	femur (100)
			leg II (73.1)	femur
				tibia (31.6)
				tarsum
				indet (5.2)
			leg III (19.2)	femur (80)
				tibia (20)
		abdomen (3.6)		
		hemelytre (3.6)		
SS	*H. skorikowi*	legs (75)	leg I (33.3)	femur (100)
			leg II (33.3)	femur (100)
			leg III (33.3)	tarsum
		head (25)		
TV	*E. infundibulifera*	abdomen (100)	segment II (59.3)	
			segment III (29.6)	
			segment IV (7.4)	
			segment V (3.7)	
	H. skorikowi	legs (87.1)	leg I (3.7)	femur (100)
			leg II (63.0)	femur
				tibia (29.4)
			leg III (33.3)	femur
				tibia (22.2)
		abdomen (6.5)	segment V (50)	
			indent (50)	
		head (3.2)		
		pronotum (3.2)		

Discussion

Differential infection between native and invasive corixids

T. verticalis is a highly successful invader in coastal wetlands of higher salinities in the southern Iberian Peninsula (Rodriguez-Perez et al., 2009; Van De Meutter et al., 2010; Guareschi et al., 2013). Although information remains limited, its ability to outcompete native corixids at high salinities seems to be related to its high fecundity and a capacity to complete several generations a year. Furthermore, the eggs and nymphs of some native corixid species do not seem resistant to such high salinities (J.A. Carbonell & C. Coccia, unpublished data). The present study supports the hypothesis that the much lower relative abundance of *T. verticalis* in temporary wetlands of lower salinity may be caused by their susceptibility to harmful parasitic mites, which are absent in the saline wetlands. We have shown that *T. verticalis* was not infected by water mites in saline wetlands, where *T. verticalis* is often the only corixid species recorded.

It is a widespread pattern that species richness of invertebrates decreases at higher salinities in Mediterranean wetlands (e.g. Frisch et al., 2006; Waterkeyn et al., 2008), and adult *Eylais* mites cannot tolerate the high salinities in the areas where *T. verticalis* is found to be dominant (V. Céspedes, A.J. Green & M.I. Sánchez unpublished data). Although we cannot rule out the possibility that the absence of mites from fish ponds is also related to the permanent hydroperiod and/or the high density of fish, decapod shrimps or other predators (Kloskowski et al.. 2009), our results from temporary wetlands support a strong salinity effect. In Generalized Linear Models, a negative partial correlation between salinity and prevalence was detected for both mite species. *Hydrachna* was particularly rare at higher salinities, so *T. verticalis* may encounter this parasite much less than *Eylais*, which was much more prevalent at higher salinities. However, date was confounded with salinity in our dataset because the temporary wetlands dry out in summer, so that a difference in phenology between mite species may be more important than a difference in salinity tolerance.

In temporary wetlands, we recorded consistently higher levels of parasitism by larval water mites in *T. verticalis* compared with *S. lateralis* and *S. scripta*, both for *H. skorikowi* and for *E. infundibulifera*. There is a clear pattern of consistently higher prevalence in *T. verticalis* for *E. infundibulifera*. In contrast, our Generalized linear Model analyses suggest that the greater prevalence of *H. skorikowi* in *T. verticalis* is only clear for *S. lateralis*, and it would as yet be premature to conclude that this mite favours *T. verticalis* as a host compared to all native species.

Both mite species recorded are obligate parasites of water boatmen (Heteroptera: Corixidae) (Stevens & Greven, 1999; Reilly & McCarthy, 1991). *H. skorikowi* is a palearctic species, so if it generally prefers *T. verticalis* as a host, this would be a case of parasite acquisition in which the exotic species becomes the preferred host compared to native ones. *E. infundibulifera* has been found in Europe (including the Iberian Peninsula), Asia and North America. Such cosmopolitan parasites are usually considered as acquired (Torchin et al., 2003; Prenter et al., 2004; Mastitsky et al., 2010), since it is much more likely that they have reencountered the parasites in the invaded area than that they were introduced with the alien host (Mastitsky et al., 2010). However, given the low prevalence of *E. infundibulifera* in native corixids, we cannot yet rule out the possibility that it has been introduced with *T. verticalis*. Studies of mite parasitism in corixid communities in parts of Iberia where *T. verticalis* has not yet arrived would shed light on this question. The means by which *T. verticalis* arrived on the peninsula are unknown, as is the date of arrival (Rodríguez-Pérez et al., 2009; Guareschi et al., 2013).

We are unaware of any other case in which an exotic insect in Europe has been shown to be more infected by parasites than native hosts. When an introduced species is a suitable host for a native parasite, this can seriously impact the exotic hosts, but can also amplify the infection ("spillback" from exotic to native species) with effects for native species at both the host individual and population level (Daszak et al., 2000; Tomkins & Poulin 2006). At the current stage of *T. verticalis* invasion our results provide no evidence of parasite spillback, but it remains a potential risk given the density and reproductive potential of the exotic host, high susceptibility for parasites and the high reproductive potential of parasites, all factors affecting the probability of spillback (Hershberger et al., 2010, Paterson et al., 2013). We can expect the opposite to the dilution effect hypothesis, which predicts that the introduction of a less competent host species may reduce infection prevalence in the native host (Telfer et al., 2005). Moreover, given the likely high dispersal abilities of *T. verticalis* (Guareschi et al., 2013), this species may enhance dispersal of mites and their introduction into new environments, as has been suggested for epibiotic mites infecting the invasive crab *Eriocheir sinensis* Milne-Edwards, 1853 (Normant et al., 2013).

Differences in parasite susceptibility observed in this study between native and alien corixids may be related to several factors. Firstly, hosts that rarely co-occur with mites in nature may be more susceptible to parasitism when spatial and temporal barriers are removed (Smith & McIver, 1984a). This can apply to invasive species which represent new hosts for native parasitic fauna. Increased susceptibility of hosts to new parasites related to a lack of co-adaptation ("naïve host syndrome", Mastitsky et al., 2010) has been reported for a wide range of parasites (Alderman et al., 1987, Burreson et al., 2000).

Alternatively, the increased susceptibility of *T. verticalis* to parasites may be caused by the differential level of sclerotization among hosts. Dark colour indicates a higher degree of sclerotization in water boatmen (Bennett, 1993). The light aspect of *T. verticalis* compared with the darker *S. lateralis* and *S. scripta* suggests that the exotic corixid is less sclerotized, and that mites could perforate the integument of *T. verticalis* with less difficulty. The higher surface area susceptible to attachment (i.e., number of body regions in which mites were found) in *T. verticalis* compared to *S. lateralis* and *S. scripta* supports this hypothesis. Bennett (1993) showed that a smaller susceptible area for attachment in sclerotized *Cenocorixa bifida*

(Hungerford, 1926) resulted in reduced overall susceptibility to *Eylais euryhalina* Smith, 1986 compared to the unsclerotized *C. expleta* (Uhler, 1895). In laboratory conditions, when equally exposed to water mites, 90% of *C. expleta* and 25% *C. bifida* were infected.

Another possibility is that biological and ecological factors affecting spatial distribution of the hosts would differentially expose them to water mite infection. Field observations (C. Coccia, personal observation) suggest that *T. verticalis* is more concentrated in the shallowest parts of ponds, where it may be more exposed to mites. Mite larvae are positively phototactic and swim to the water surface in search of hosts (Lanciani 1969), and it is also possible that *T. verticalis* coincides more often with the larvae within the water column. For example, *S. lateralis* feed more on benthic chironomid larvae (Tawfik et al., 1990) while *T. verticalis* may feed more on zooplankton in the water column (Wurtsbaugh, 1992; Simonis, 2013).

Behavioural factors may also play a role in our results. Some corixid species are able to limit infestations by eating larval mites (Lanciani, 1985) or by defensive behaviours (Smith and McIver 1984b). Moreover, physiological aspects related with the ability of some species of corixids (*Sigara*) to impede engorgement of *Hydrachna* and *Eylais* species by reacting against the stilistoma of the mite (sometimes provoking the death of the parasite) (Davids, 1973) may also partly explain our results. Experimental infection with equal exposure and behavioural tests would be necessary to discern between these hypotheses.

Effect of host sex, host size and attachment site

Like Smith (1977), we found no differences between sexes in parasite infection. Sexual preferences may be related with differences between sexes in size, time of emergence, differential exposure caused by different behaviors or different dispersal patterns. The low prevalence in *C. affinis* (by far the largest host species 7.2-10.5 mm Nieser et al., 1994) and high prevalence in *T. verticalis* (the smallest species in our study) suggests the mites show no preference for larger host species. Although host size has previously been shown to influence parasite growth for *Hydrachna* and *Eylais* species (Davids & Schoots 1975), we didn't find differences in parasite size when infecting native and invasive species, suggesting that all hosts are equally suitable for engorgement. Although *T. verticalis* is significantly smaller that native species this difference is perhaps too small (<14% difference in length) to have a noticeable effect on the mites. Bennett (1993) found fully engorged mites preferentially on lightly sclerotized corixids and rarely on highly sclerotized species, which suggests that parasite growth also depends on host sclerotization. Therefore, the low sclerotisation of *T. verticalis* may compensate for its smaller size. The size of the parasite relative to it host influences the degree of the damage it can induce (for example in fecundity, Davids & Schoots, 1975). So for a given parasite size, we can expect more damage in a smaller host such as *T. verticalis*. However, laboratory growth experiments would be necessary to confirm that mite growth does not vary between host species.

The precise attachment site is relevant to the understanding of the effects of mites in their hosts, and to host-parasite coevolutionary interactions (Bennett & Scudder, 1998). The attachment site for both mite species was highly species-specific and reflects different life histories. *Eylais* larvae are semi-aquatic requiring an air supply to survive, and are therefore restricted to areas such as under the wings, tergites, underside of the elytra and hemielytra (Lanciani, 1969, Nielsen & Davids 1975, Davids et al., 1977). In our study they were invariably found attached to the abdominal tergites under the wings, which is likely to damage flight musculature (Smith, 1988). In contrast, Hydrachnidae larvae are strictly aquatic and can use dissolved oxygen in the water. Therefore they can be found attached to all surfaces of the host (Harris & Harrison, 1974), and in our study they were observed on the wings, head and legs. We did not find any preference between the right and left side of the host. In some species of *Sigara* the right hemelytron is more infected because it overlaps the left one (Davids, 1973), although this is not a consistent result (Mitchell, 1968).

Ecological impact of mite infection and consequences for *T. verticalis* invasion

Mites have the capacity to have a major influence on the extent of invasion by *T. verticalis*. Smith (1977) previously showed that the spatial distribution of two sympatric water boatmen was determined by the presence of water mites, which exclude one of them at lower salinity. In many host-parasite systems, values of prevalence exceeding 10%, as in our study for *T. verticalis*, are enough to exert a negative influence on host density (Hall et al., 2011). Moreover, total prevalence and intensity of mites recorded in our study were probably underestimates. We often found brownish spots in the point of attachment of larval mites in all corixid species, indicating the previous presence of parasites (Figure 5). In fact, in *Arrenurus* Dugès, 1833 species these marks have been used to accurately estimate the number of larvae that had been attached to the host (Lanciani, 1979).

Mite-induced reduction in survival has been demonstrated for a variety of host-parasite associations (Lanciani, 1982, 1986). Numerous studies have shown that parasitism by mites adversely affects insects (Smith, 1988). Fernando and Galbraith (1970) reported disappearance of gerrid populations heavily infected by water mites. Hence mites have the potential to cause local extinctions of *T. verticalis*.

Specific information about the ecological impacts of *E. infundibulifera* and *H. skorikowi* larvae are lacking. However, the existing literature suggests that negative effects of infection can be expected at both the host individual and population levels. Both *Eylais* spp. and *Hydrachna* spp. experience a dramatic increase in size during the larval phase. The *Eylais* genus includes the largest species of all water mites (Lanciani, 1971) and some species of *Hydrachna* can increase their volume by 600 times from birth (Davids, 1973). Enlargement of the larvae is correlated with the time spent on the host, and in these genera the duration of the larval engorgement period can be very long (several months, Bennett, 1993). Therefore, the time of the parasitic phase together with the size reached by the larvae in our study are both expected to negatively impact the hosts.

On the other hand, water mite larvae have been shown to destroy host tissue (Abro, 1982) and adversely affect flight musculature (Smith, 1988), consequently affecting the host's flight ability (Gillies & Wilkes 1972). The ability to fly and disperse is fundamental for the survival of aquatic insects living in temporary habitats, such as corixids (Savage, 1989; Boda & Csabai, 2009). Larval *Hydrachna* spp. and *Eylais* spp. infecting aquatic Hemiptera can also dramatically decrease fecundity by reducing egg production (Davids & Schoots, 1975).

Other reproductive effects caused by mites in corixids include delayed maturation of the host (Lanciani, 1975), reduction of nymphal growth (Lanciani & May, 1982) that may affect competitiveness and survival (Martin 1975), and reduction of male mating success (Forbes, 1991a, b, Forbes & Baker 1991). Deutonymphs and adults of *H. skorikowi* have also been shown to feed on eggs of water boatmen (Stevens & Greven, 1999), as have other *Hydrachna* species (Davids, 1979). High intensity of infection can induce mortality (Lanciani, 1975), impacting at the population level. In this study, the values of infection intensity for *T. verticalis* (up to 7) are among the highest recorded for corixids. All these effects can potentially be stronger in the exotic species under the naïve host syndrome (Mastitsky et al., 2010).

Indirect effects can also be expected. *Eylais* and *Hydrachna* nymphs feed on Cladocera, where *T. verticalis* also occur and may compete for these prey (Simonis, 2013). Since water mites can be very abundant (up to 13,000 eggs per female over a period of 12 months have been reported for *Eylais discreta*, Davids 1973), this may result in competition for food between mites and corixids. Further research should focus on the ecological impact of mites on the *T. verticalis* invasion and its interactions with native corixids, using naturally infected populations in combination with experimental laboratory infections.

Conclusion

T. verticalis showed consistently higher infection levels by water mite larvae compared with the native corixid hosts *S. lateralis* and *S. scripta*. We found evidence that the invasion success of *T. verticalis* in natural wetlands of low salinity has been limited owing to a higher susceptibility to parasites compared with native species. Since water mites strongly reduce reproductive success and increase mortality at high intensities, they are likely to play a key role in driving the outcome of ecological interactions between the invasive and the native species. This study suggests that mites may prevent *T. verticalis* from colonizing low salinity wetlands or outcompeting the native corixids there. As the invader spreads across Europe in future decades, the mites may play a vital role in conservation on native insect diversity.

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Supplementary Materials

Table 1. Details of *Trichocorixa verticalis* inspected for water mite larvae in Veta la Palma fish ponds (G3, A3 and A7) during May, June and July 2011. Note pond A3 has lower salinity than the other ponds because it is managed for shrimp production. No mites were recorded. Adult mites were not recorded in the area during a previous study of the invertebrate community (Rodríguez-Pérez & Green, 2012).

Ponds	**Date**	**Salinity**	***n***
A7	19/05/11	11.9	22
G3	19/05/11	24.5	37
A3	19/05/11	4.3	246
G3	08/06/11	25.8	26
A3	08/06/11	5.8	68
A7	13/07/11	21.4	13
A3	13/07/11	8.1	497

Chapter 4

Can differential predation of native and alien corixids explain the success of *Trichocorixa verticalis verticalis* in the Iberian Peninsula?

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Abstract

Invasive species represent an increasing fraction of aquatic biota. However, studies on the role and consequences of facilitative interactions among aliens remain scarce. Here we investigated whether the spread of the alien water boatman *Trichocorixa verticalis verticalis* in the Iberian Peninsula is related to reduced mortality from predation compared with native Corixidae, especially since *T. verticalis* co-occurs with the invasive fishes *Gambusia holbrooki* and *Fundulus heteroclitus.* All three invaders have a common native range in North America and are widespread in and around Doñana in SW Spain. Using laboratory experiments, we compared the predation rates by the two exotic fish and native Odonata larvae on *Trichocorixa* and the native *Sigara lateralis.* We found no evidence to suggest that *T. verticalis* suffers lower predation rates. However, when both corixids were mixed together, predation of *T. verticalis* by Odonata larvae was higher. Odonata larvae were size-limited predators and the proportion of corixids ingested was positively correlated with mask length. Since *T. verticalis* is smaller than its native competitors, this may explain their higher susceptibility to predation by Odonata. This may be one of various factors explaining why *T. verticalis* is particularly dominant in saline habitats where Odonata are rare, while it is still scarce in fresh waters.

Introduction

Freshwater habitats have been subjected to massive species introductions from multiple sources for decades (Gherardi et al., 2008). As a consequence, in many of these systems, alien organisms are now a significant part of their biota (Cohen, 2002). Iberian inland waters, for example, host *ca* 73 non-indigenous freshwater species (Garcia-Berthou et al., 2007), belonging to a variety of taxa such as molluscs, crustaceans and fishes (Cobo et al., 2010).

Although negative interactions between species have been considered to be among the major drivers of biological invasions, only recently have studies highlighted the role of facilitative interactions among exotic species (Simberloff & Holle, 1999; Richardson et al., 2000). According to Courchamp et al. (2000), the co-occurrence of exotic predators and exotic prey can heavily impact native prey by hyperpredation. Adams et al. (2003) demonstrated how the invasion of the bullfrog *Lithobates catesbeianus* in North America was facilitated by the invasive bluegill sunfish *Lepomis macrochirus*, as the latter preys on macroinvertebrates that in turn prey on bullfrog tadpoles. Such positive interactions are thought to be widespread and important in exacerbating the problem of invasion (Simberloff & Holle 1999), but studies that evaluate these interactions are still scarce (Richardson et al., 2000; Tecco et al., 2006), particularly for vertebrates (but see Adams et al., 2003).

In this study we experimentally investigated the role of predation by exotic predators (the eastern mosquitofish *Gambusia holbrooki* and the mummichog *Fundulus heteroclitus*; hereafter *Gambusi*a and *Fundulus*, respectively) and native predators (dragonfly larvae) on the invasive boatman *Trichocorixa verticalis verticalis* (hereafter *T. verticalis*) and the native, co-occurring boatman, *S. lateralis* (hereafter *S. lateralis*), in the Iberian Peninsula. *T. verticalis* (Fieber 1851) (Heteroptera: Corixidae) is native to North American saline and freshwater habitats but now also occurs in South Africa, Iberia and Morocco (Jansson & Reavell, 1999; Kment, 2006; L'Mohdi et al., 2010). In Europe, following its initial detection in the Algarve in Portugal (Iberian Peninsula) in 1997, the species has spread along the Iberian Atlantic coast (Sala & Boix 2005) and into the Guadalquivir delta and surrounding parts of SW Spain. Bio-climatic models predict future spread across Europe and the Mediterranean region (Guareschi et al., 2013). Established populations of *T. verticalis* have been found at several sites in and around Doñana in the Guadalquivir delta, where it is the most abundant corixid in saline wetlands (Rodríguez-Pérez et al., 2009; Van de Meutter et al., 2010). Despite their dominance at higher salinities, they do not show higher halotolerance as adults than native corixids (Van de Meutter et al., 2010; Coccia et al., 2013).

In its native area, *T. verticalis* co-occurs in rivers, brackish pools, potholes, and salt marshes with the above two euryhaline fish species (*Gambusia* along the east coast of USA from New Jersey to Florida and *Fundulus* along the North American Atlantic coast from Canada to Florida, see Sala & Boix, 2005). Various studies illustrate the coexistence of this corixid species with one of these fish species in the same waterbody within the native range (e.g. Harrington & Harrington, 1972; Cherry et al., 1979; MacKenzie, 2005; Rochlin et al., 2011; J. Simonis, pers. comm. 2014). These fishes were introduced to the Iberian Peninsula from North America around 1921 and 1970 respectively (Cobo et al., 2010). They now occur in a multitude of aquatic habitats during all the hydrological cycle in Doñana, where they are far more abundant than any native freshwater fish (Moreno-Valcárcel et al., 2013). Both species have been shown by gut content analysis to include corixids in their diet in permanent saline fish ponds in Doñana, where Odonata are absent or only recorded at low densities (Pyke, 2005; Dreamer-John, 2012; Rodríguez-Pérez & Green, 2012). In contrast, Odonata larvae represent the most important predators of corixids in temporary ponds in Doñana, where the invasive and native corixids co-exist but fishes rarely occur (Florencio et al., 2009; Rodríguez-Pérez et al., 2009).

As exotic predators can have dramatic effects on both invasive and native prey (Lodge, 1993; Knapp et al., 2001), only organisms with specific and effective anti-predatory mechanisms are likely to coexist with them (Sih et al., 2010). Although some studies have shown native prey to possess better defenses against native than alien predators (Gomez-Mestre & Díaz-Paniagua 2011), prey species may also undergo rapid evolutionary change to counter a novel predation pressure (Griffin, 2004; Boyero, 2011). However, predator detection has a cost and performance can be lost with similar speed if the predator is removed (Strauss, Lau & Carroll, 2006).

We hypothesized that alien predators (fish) and those native to Doñana (Odonata larvae) would have different interactions with the alien (*T. verticalis*) and native (*S. lateralis*) corixids. Specifically, we tested the following hypotheses: i) predator responses to different prey depend on co-evolutionary history; thus, the exotic predators *Fundulus* and *Gambusia* are more successful at detecting and catching *T. verticalis*, while Odonata are more successful at detecting and catching *S. lateralis*; and ii) differences in predation also depend on predator and prey size. We discuss the evidence that facilitative interactions can account for *T. verticalis* success in southern Iberia and whether the scarcity of Odonata larvae in permanent, saline habitats in Doñana could be a factor promoting its dominance in this area (Rodríguez-Pérez et al., 2009; Van de Muetter, Trekels & Green, 2010).

Material & Methods

Methods

Adults of *Fundulus* and *Gambusia* were collected during spring 2012 using either minnow traps or a D-framed pond net (500 μm mesh; 16 × 16 cm), respectively. Native Odonata larvae and adults of *T. verticalis* and *S. lateralis* were sampled using the same D-framed pond net. Experiments were run from 28 March to 16 May 2012. *Fundulus* and *T. verticalis* were obtained from Veta la Palma fish ponds within Doñana Natural Park, *Gambusia* from an artificial pond called "pozo salinas" located in the stabilized sands (a system of stable dunes formed by marine drift during the Holocene period) in the west part of Doñana Biological Reserve and *S. lateralis* were sampled in two artificial ponds within the garden of the Cartuja Monastery (within the city of Seville). Finally, Odonata larvae were collected in artificial ponds either within Doñana National Park (FAO pond) or the Cartuja Monastery (see Serrano et al., 2006 for position of the Doñana sites).

Water conductivity varied between sites, being 32 - 36 mS cm^{-1} in Veta la Palma, and 1.8 - 5 mS cm^{-1} in the other ponds.

After collection, predators and prey were placed separately in plastic bags (a maximum of 4 fishes per bag) filled with water from the collection site and transported to the laboratory within thermally insulated polystyrene boxes to minimise thermal fluctuations. Once in the laboratory, each predator was transferred to its own 6-L aquarium (27 x 17 x 18 cm), containing water at conductivities that resembled those of sites from which they were collected [17 mS $cm^{-1}$for *Fundulus*; 5 mS cm^{-1} for *Gambusia* (ca. 11 cm depth); and 2 mS cm^{-1}, for Odonata larvae (ca. 6 cm depth)]. Water from the collection point was used for the treatments of 5 and 2 mS cm^{-1}, whereas for high conductivity treatments, water was prepared by mixing pond water (32 - 36 mS cm^{-1}) with dechlorinated tap water until the required conductivity of 17 mS cm^{-1} was obtained. As we did not test for turbidity effects, all treatments were conducted in clear water. Aquaria were provided with sand and were aerated for the fish treatments. Predators were acclimated for 24 h before predation experiments; this period is considered sufficient to allow fishes to explore the aquaria (Primavera 1997). No food was provided to predators during these 24 h.

Experiments were conducted in a climatic chamber set at 15ºC under a 12 h: 12 h D:L regime. Ten corixids (balanced for sex when possible) were added to each aquarium containing a predator individual. When fishes were used as predators, six treatments were created by crossing corixid species (*T. verticalis* alone, *S. lateralis* alone and both species together) and refuges (with and without an artificial plant, Supplementary Fig. 1a,b). In the case of Odonata larvae, only three treatments were employed (the three corixid combinations) as aquaria were always provided with artificial plants to provide a perch for the larvae. Five replicates of each treatment were used for each fish species and 10 replicates for dragonflies. The first check for surviving corixids was made after 6 h. Thereafter, aquaria were checked every 24 h. At each check, live corixids were retrieved using a hand net, counted, identified to species and visually sexed. After that, the corixids were returned to their aquarium. Consumed corixids were not replaced. After a maximum of 72 h, predators were retrieved from each aquarium. Each predator was used only once and sacrificed under licence after the experiment, using anaesthetic. Fishes were weighed using a ALC_2100.1 balance (Sartorious Ltd, U.K.) and the length of each individual was measured using a plastic millimeter sheet. Mean predator sizes (± SE) were as follows: 6.9±0.13 cm for *Fundulus* and 3.8±0.04 cm for *Gambusia.* The mean lengths (± SE) of masks of Odonata larvae (4.03±0.14 mm) were measured on images taken with a digital camera (AxioCam Icc1) connected to a Zeiss microscope (Discovery V8) (Supplementary Table 1 and Fig. 2). Odonata larvae were mainly (70%) final instars, and the remaining 30% were mid instars (according to Askew 1998). Prey size was not measured, as it would have involved a strong handling stress that may have altered corixid behaviour. The typical lengths were 5-6 mm for *S. lateralis* (Nieser et al., 1994) and 3.5-5.4 mm for *T. verticalis* (authors'unpublished data).

Data analysis

We quantified the proportion of corixids (either *S. lateralis* or *T. verticalis*) that were eaten by predators (Proportion of Corixids Eaten, PCE = 1 – final/initial number of corixid individuals), for each predator (*Fundulus*, *Gambusia*, and Odonata) and for each corixid treatment (single species vs. both species together). Although we used larvae of several dragonfly genera of the families Libellulidae and Aeshnidae [*Orthetrum* spp. (*n*=10); *Aeshna* spp. (*n*= 19); *Sympetrum* spp. (*n*= 10); and *Crocothemis* spp. (*n*=1)], we pooled the data for final analyses presented because results were the same as when using only the most frequent genus (*Aeshna* spp., other taxa could not be analysed separately because there were not enough individuals, see Supplementary Table 2). Initial models showed that there were no significant differences in PCE for fish between treatments with and without an artificial plant (results not shown). Thus, these treatments were pooled for further analyses comparing PCE between predator and prey species.

We used general linear models (GLMs) followed by Tukey tests to compare, for each corixid species separately, the effect of predator identity, corixid treatment and their interaction on PCE, which was arcsin square root transformed to improve normality and homogeneity of variances (tested with Shapiro-Wilk's and Levene's tests, respectively). Given the differential consumption rates between *Fundulus* (the most voracious), *Gambusia* and dragonflies, we initially calculated PCE for several experimental time intervals (6 and 24h for *Fundulus*; 6, 24, 48 and 72h for *Gambusia* and dragonflies), but we present results only for the final time (24h for *Fundulus* and 72h for *Gambusia* and dragonflies) because (1) *Gambusia* and dragonflies started eating after 24h and (2) results of analyses were the same between 6 and 24h for *Fundulus* and between 48 and 72h for *Gambusia* and dragonflies.

As the interaction between predator identity and corixid treatment was significant for *T. verticalis*, we then used separate GLMs for each predator species to further explore effects of corixid treatment on PCE, for each corixid species separately. In these analyses we included the log (x+1) transformed predator size (for *Fundulus* and *Gambusia*) or dragonfly mask length as a covariate. Finally, we compared PCE between corixid species for the 'both species together' treatment with one-way ANOVA for each predator separately.

Results

Predator identity had a significant effect on PCE (Table 1), although results differed slightly for each corixid species. For *S. lateralis*, *Fundulus* showed the highest predation rate followed by Odonata larvae and *Gambusia*. For *T. verticalis*, *Fundulus* and Odonata were equally efficient, and *Gambusia* was again the least efficient predator (Fig. 1). Corixid treatment had no effect on PCE, but the interaction between predator identity and corixid treatment was significant for *T. verticalis* (Table 1); differences between corixid treatments were significant for Odonata ($P < 0.05$), which consumed significantly more *T. verticalis* when *S. lateralis* was also present, but not for the other predators (Fig. 1).

When the effect of different predators was analyzed separately, there was no significant effect of either corixid treatment or predator body length for *Fundulus* and *Gambusia* (results not shown). For Odonata larvae, corixid treatment had no effect for *S. lateralis*; however, corixid treatment did have a significant effect for *T. verticalis*. Mask length had a positive effect on PCE for both species (Table 2): dragonflies with larger masks ate more corixids (Fig. 2). When both corixid species were together, there were no significant differences between the number of *S. lateralis* and *T. verticalis* eaten by any predator (*Fundulus*: $F_{1,42} = 0.06$, $P = 0.78$; *Gambusia*: $F_{1,38} = 1.28$, $P = 0.27$; dragonflies: $F_{1,38} = 3.50$, $P = 0.069$); however, the marginal result for dragonflies shows they tended to eat more *T. verticalis* individuals (Fig. 3). Hence, the predation efficiency of Odonata larvae increased with their size, and was marginally influenced by prey size.

Table 1. Results (degrees of freedom, sum of squares, F-statistic and p-value) of GLMs showing the effects of predator identity (*Fundulus heteroclitus*, *Gambusia holbrooki* or dragonflies larvae), corixid treatment (single species or both species together), and their interaction, on the proportion (arcsin sqrt transformed) of corixids eaten by predators.

Source of variation	df	SS	*F*	*P*
Sigara lateralis				
Predator	2	14.51	36.93	< 0.0001
Corixid	1	0.01	0.03	0.87
Predator × Corixid	2	0.54	1.38	0.26
Error	85	16.70		
Trichocorixa verticalis				
Predator	2	17.14	41.17	< 0.0001
Corixid	1	0.12	0.60	0.44
Predator × Corixid	2	1.49	3.58	0.032
Error	85	17.69		

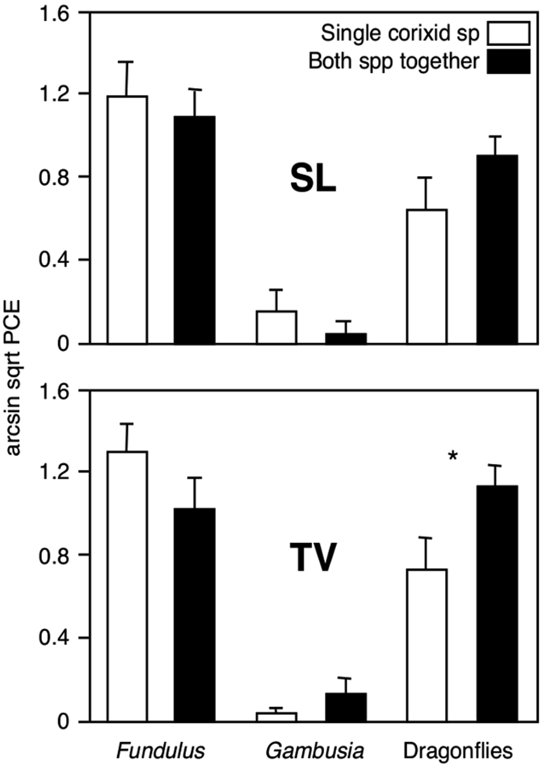

Figure 1. Arcsin sqrt-transformed mean ± SE proportion of corixids eaten (PCE = 1 - final/initial number of corixid individuals) for each corixid species (SL, *Sigara lateralis*; TV, *Trichocorixa verticalis*), by different predators (*Fundulus heteroclitus*, *Gambusia holbrooki*, and Dragonflies), and in two different corixid treatments (single species vs. both species together). Asterisks indicate significant differences in the PCE of TV by Dragonflies compared to the other predators when SL was also present.

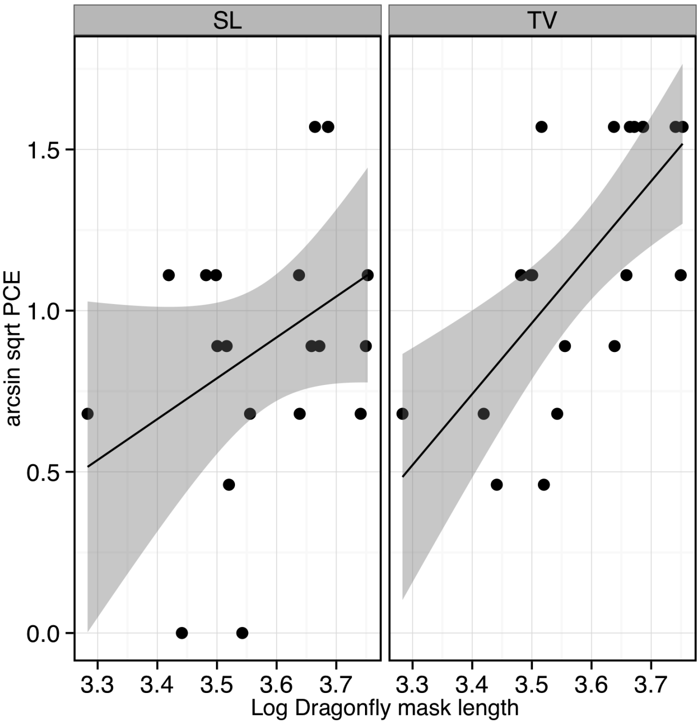

Figure 2. Linear regression (+ 95% confidence interval) between log-transformed dragonfly larva mask length and arcsin sqrt-transformed proportion of corixids eaten (PCE); SL, *Sigara lateralis (P* = 0.11*, R^2* = 0.13*)*; TV, *Trichocorixa verticalis (P* = 0.001*; R^2* = 0.46*).*

Table 2. Results of GLMs showing the effects of corixid treatment (single species or both species together) and log (x+1) transformed mask length of the arcsin sqrt transformed proportion of corixids eaten by Odonata larvae.

Source of variation	**df**	**SS**	**F**	***P***
Sigara lateralis				
Corixid	1	0.48	2.82	0.10
Dragonfly mask length	1	0.93	5.47	0.027
Error	27	4.57		
Trichocorixa verticalis				
Corixid	1	1.50	10.95	0.003
Dragonfly mask length	1	1.41	10.36	0.003
Error	27	3.69		

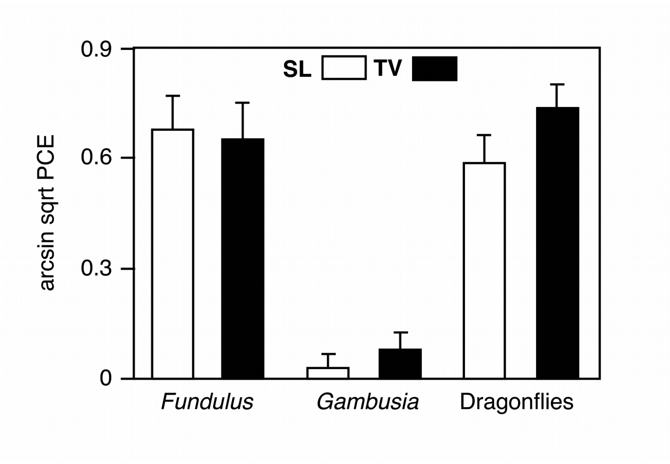

Figure 3. Arcsin sqrt-transformed mean ± SE proportion of corixids eaten (PCE) of each species (SL, *Sigara lateralis*; TV, *Trichocorixa verticalis*) in the 'both species together' treatment.

Discussion

In a multiple invasion context, where invaders at different trophic levels co-exist, several scenarios are possible. For example, alien predators may prefer native prey, native predators may prefer alien prey, or predators may easily detect/recognize prey because they share a co-evolutionary history (Sih et al., 2010).

Our results indicate that the invasive *Fundulus* and the native Odonata larvae are considerably more efficient than the invasive *Gambusia* in the rate of consumption of corixids. However, the only evidence to suggest that predators distinguish between alien and native corixids was on the basis of body size. *T. verticalis* body mass is 35% lower than that of *S. lateralis* (Coccia et al., 2013), and our results suggest that for that reason they suffer higher predation by Odonata, and that this may help explain *T. verticalis* dominance in saline waters in SW-Spain.

We found no evidence to suggest that corixids show different predator avoidance behaviour depending on whether they encountered predators sharing a common native range or predators of a different origin. However, our study did not allow us to distinguish between the existence of anti-predator responses in corixids vs. prey detection/capture mechanisms in predators, and we did not collect behavioural data. The absence of an efficient anti-predator response can lead to species decline (Courchamp et al., 2000), and the apparent inefficiency of anti-predator responses contradict field data confirming the co-occurrence of both exotic fishes and corixids in Doñana (Van de Meutter, Trekels & Green, 2010; authors' unpublished data). Under confined laboratory conditions and high water clarity, it is likely that predator efficiency was greater than that in the field, where habitats are more diverse with reduced spatial or temporal overlap in habitat use between predator and prey, and turbidity can be high. For example, Kelts (1979) showed an increased rate of consumption of *T.verticalis* var *sellaris* by *Fundulus* when algae were removed from aquaria.

The plastic plant we used as a refuge had no similar effect and was probably insufficient to hinder prey detection (Supplementary Fig. 1a,b).

In Doñana, *Fundulus* and *Gambusia* occur in different types of habitat (see Supplementary Table 3), however *Fundulus* prefers saline waters, and is generally absent in fresh or brackish ponds, which are inhabited mainly by *Gambusia* (Moreno-Valcárcel et al., 2013). *Trichocorixa* and *Sigara* often co-occur in sites of 3-15 mS cm^{-1} (Van de Meutter at al., 2010), whereas *Trichocorixa* is the dominant corixid at higher salinities and *Sigara* at lower salinities. Thus, we might expect a strong effect of *Fundulus* for both corixids at salinities between 9-23 mS cm^{-1} where *Fundulus* dominates (Moreno-Valcárcel et al., 2013). However, in most of the sites were *T. verticalis* was found with native corixids within the Doñana area, *Gambusia* was the only fish present (Van de Meutter, Trekels & Green, 2010). At low conductivities (0.3-8.5 mS cm^{-1}), Moreno-Valcárcel et al. (2013) also found *Gambusia* to be the dominant species. The low observed predation rate by *Gambusia* in our experiments suggests that the predator-prey interaction between *Gambusia* and both the corixids is less important than that of *Fundulus*, a finding in agreement with field data showing that *Gambusia* ate mainly copepods and relatively few corixids (Dreamer-John, 2012). In contrast, our results suggest that *Fundulus* and Odonata larvae may have a major impact on the invasive corixids in permanent saline waters and low salinity ponds respectively.

Permanent saline waters in SW Spain are often inhabited by both *Fundulus* and *T. verticalis* (Sala & Boix, 2005). It has been suggested that the unpalatability of *T. verticalis* late instars and adults may reduce predation by *Fundulus* in North America (Campbell & Denno, 1978; Kelts, 1979), but our observations do not support this. The extent of predation on *T. verticalis* by *Fundulus* is likely to partly depend on the availability of alternative prey such as copepoda, isopoda and decapod shrimps (Dreamer-John, 2012). Furthermore, the distribution and abundance of *Fundulus* is likely to be limited by its own predators, such as larger fish and piscivorous birds, both of which are present at high density in saline parts of Doñana where *T. verticalis* is dominant (Rendón et al., 2008, Rodríguez-Pérez & Green 2012). Additionally, co-occurrence with *Fundulus* may also be related to *T. verticalis* preferences for shallow, vegetated waters (authors personal observation) avoided by fish (Kelts, 1979; Lewin et al., 2004). At the same time, the osmoregulatory ability of the invasive corixid at the egg and nymph stage together with its high fecundity and the absence of parasitic infections might be some important determinants of its success at higher salinities (authors' unpublished data).

In temporary ponds, corixids arrive *en masse* in spring to reproduce, and the absence of fish maximises nymphal survival (Brown, 1951). Odonata larvae and both corixid species are often found together in Doñana ponds, especially during late spring (Florencio et al., 2009; Rodríguez-Pérez et al., 2009). Odonata larvae are top predators in temporary aquatic habitats, able to feed on different prey ranging from invertebrates (Hopper, 2001) to small fishes (Walker, 1953). Our results illustrate how Odonata larvae are size-limited predators, and suggest that predation of *T. verticalis* by these major pond predators is increased by their smaller size compared to native *Sigara* spp. (including *S. scripta*, *S. stagnalis* and *S. selecta*, Rodríguez-Pérez et al. 2009, Van de Meutter, Trekels & Green, 2010). The predator size-prey size relationship is one of many factors that can lead to greater use of exotic species as prey by native predators (Anholt & Werner 1998). There are many previous example of native predators consuming exotic prey (King et al., 2006; Bulté & Blouin-Demers, 2008), sometimes as the dominant food items (Carlsson et al., 2009). In temporary ponds, predation by Odonata larvae may provide biotic resistance to invasion (Levine, Adler & Yelenik 2004), limiting the abundance of *T. verticalis* which frequently reproduces in temporary ponds in the native range (Brown, 1951). Selective predation can often mediate similar co-existence of similar prey (Ciros-Pérez et al., 2004).

Conclusion

T. verticalis is a successful invader in SW Spain and is particularly dominant over native corixid species in saline wetlands (Rodríguez et al., 2009, Van de Meutter, Trekels & Green, , 2010). Our findings provide no support for the hypothesis that the *T. verticalis* invasion has been promoted by lower predation rates compared to native corixids. On the other hand, *T. verticalis* may suffer particularly higher predation rates from native Odonata larvae, which may partly explain why this species is particularly successful in saline habitats where Odonata are rare. Further studies are required to compare the life histories of the alien and native corixids. Priorities for future research include direct interactions (aggression, predation) between *T. verticalis* and native heteropterans, as well the trophic ecology of *T. verticalis* and its role in invaded food webs.

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Supplementary Materials

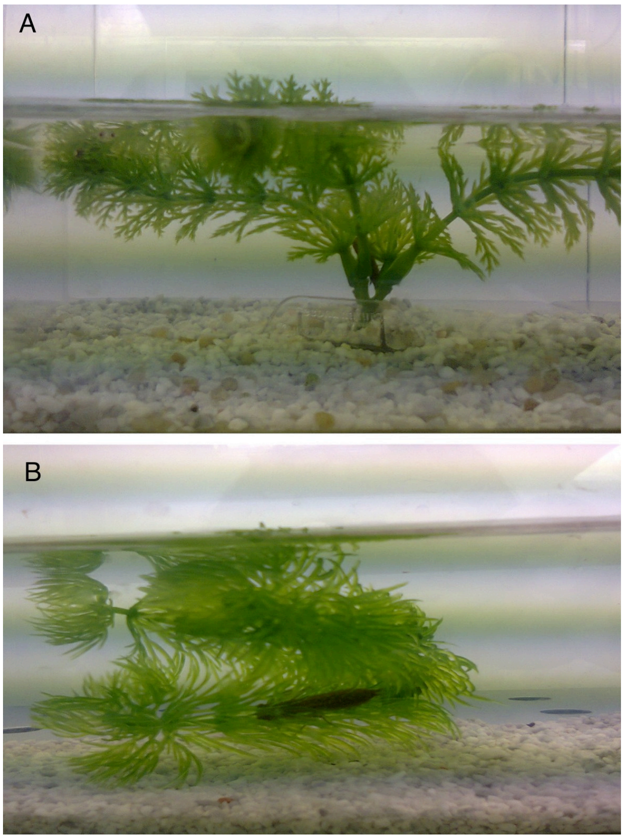

Figure 1. Artificial plant used in the experiment.

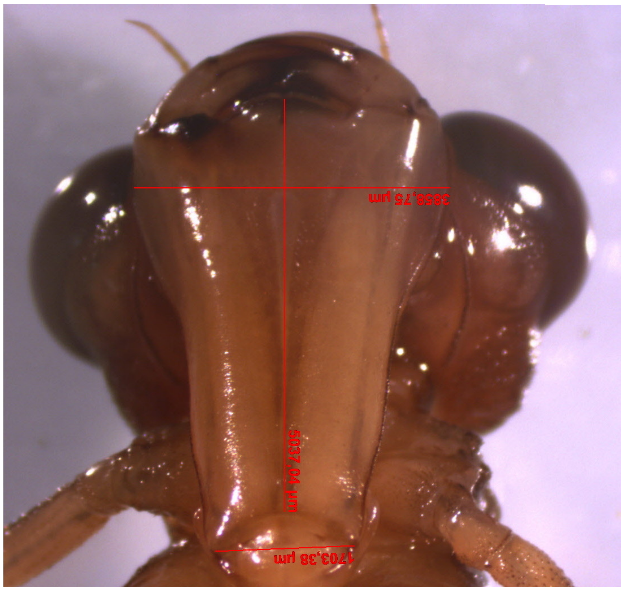

Figure 2. Example of stereomicroscope photograph of Odonata larvae mask.

Table 1. Dragonflies mask length.

N	Origen	Genus	Mask Length (mm)	Treatment
1	Cartuja M.	*Orthetrum* spp.	3.5	SL+TV
2	Cartuja M.	*Orthetrum* spp.	3.6	SL+TV
3	Cartuja M.	*Orthetrum* spp.	3.0	SL+TV
4	Cartuja M.	*Orthetrum* spp.	3.3	SL+TV
5	Cartuja M.	*Orthetrum* spp.	3.1	SL+TV
6	Cartuja M.	*Orthetrum* spp.	3.3	SL+TV
7	Cartuja M.	*Orthetrum* spp.	2.8	SL+TV
8	Cartuja M.	*Orthetrum* spp.	3.2	SL+TV
9	Cartuja M.	*Orthetrum* spp.	2.6	SL+TV
10	Cartuja M.	*Orthetrum* spp.	1.9	SL+TV
1	FAO	*Aesha* spp.	5.6	SL+TV
2	FAO	*Aesha* spp.	4.7	SL+TV
3	FAO	*Aesha* spp.	5.5	SL+TV
4	FAO	*Aesha* spp.	5.6	SL+TV
5	FAO	*Aesha* spp.	4.3	SL+TV
6	FAO	*Aesha* spp.	4.8	SL+TV
7	FAO	*Aesha* spp.	4.3	SL+TV
8	FAO	*Aesha* spp.	4.5	SL+TV
9	FAO	*Aesha* spp.	4.6	SL+TV
10	FAO	*Aesha* spp.	4.8	SL+TV
1	FAO	*Aesha* spp.	4.9	SL
2	FAO	*Aesha* spp.	5.6	SL
3	FAO	*Aesha* spp.	4.7	SL
4	FAO	*Aesha* spp.	3.5	SL
5	FAO	*Sympetrum* spp.	3.5	SL
6	FAO	*Sympetrum* spp.	3.5	SL
7	FAO	*Sympetrum* spp.	3.4	SL
8	FAO	*Sympetrum* spp.	3.4	SL
9	FAO	*Sympetrum* spp.	3.7	SL
10	FAO	*Sympetrum* spp.	3.6	SL
1	FAO	*Aesha* spp.	4.7	TV
2	FAO	*Aesha* spp.	4.1	TV
3	FAO	*Aesha* spp.	4.7	TV
4	FAO	*Aesha* spp.	4.6	TV
5	FAO	*Aesha* spp.	5.6	TV
6	FAO	*Crocothemis* spp.	3.4	TV
7	FAO	*Sympetrum* spp.	3.6	TV
8	FAO	*Sympetrum* spp.	3.6	TV
9	FAO	*Sympetrum* spp.	3.7	TV
10	FAO	*Sympetrum* spp.	3.9	TV

Table 2. Comparison of the results (degrees of freedom, sum of squares, F-statistic and p-value) of GLMs performed with all Odonata genera with those including only the most frequent genus *Aeshna* spp. on the proportion (arcsin sqrt transformed) of corixids eaten by them.

Source of variation	**Df**	**SS**	**F**	***P***
***Aeshna* spp.**				
Sigara lateralis				
Predator	2	16.132	43.737	< 0.001
Corixid	1	0.041	0.221	0.639
Predator x Corixid	2	0.044	0.120	0.886
Error	69	12.72		
Trichocorixa verticalis				
Predator	2	18.294	44.132	< 0.001
Corixid	1	0.208	1.003	0.319
Predator x Corixid	2	1.416	3.416	0.038
Error	70	14.51		
All Odonata				
Sigara lateralis				
Predator	2	14.510	36.929	< 0.001
Corixid	1	0.005	0.027	0. 868
Predator x Corixid	2	0.543	1.382	0.256
Error	85	16.70		
Trichocorixa verticalis				
Predator	2	17.138	41.167	< 0.001
Corixid	1	0.124	0.597	0.442
Predator x Corixid	2	1.491	3.581	0.032
Error	85	17.69		

Table 3. Types of habitat where *Fundulus heterolictus*, *Gambusia holbrooki*, Odonata larvae and Corixids (*Sigara lateralis* and *Trichocorixa verticalis*) occur within the Doñana area.

Habitat occurrence	***Fundulus heteroclitus***	***Gambusia holbrooki***	**Odonata larvae**	***Sigara lateralis***	***Trichocorixa verticalis***
Temporary ponds		x*	x	x	x
Permanent fresh		x	x	x	x
Small streams	x	x	x	x	x
Seasonal lakes	x	x	x	x	x
Saline fish ponds	x	x	x		x
Solar saltworks	x				x
Waterholes		x	x	x	x
Ditches	x	x	x	x	x

Chapter 5

Newly created ponds complement natural water bodies for macroinvertebrate conservation in South West Spain

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Submitted to *Freshwater Biology*

Abstract

Ecological restoration is becoming increasingly widespread to compensate for wetland loss and transformation worldwide. However, most post-restoration studies are descriptive, limited to particular taxonomic groups and fail to pinpoint underlying drivers of community assembly over time. During two consecutive inundations (2009 - 2011), we studied the macroinvertebrate communities in 32 temporary ponds constructed between 2004 and 2005 during a restoration in Doñana, SW Spain, and compared them with ten natural reference sites nearby. We compared two dominant groups of active dispersers (Coleoptera and Hemiptera), with the whole aquatic macroinvertebrate community (a mix of active and passive dispersers) to shed light on dispersal constraints during ecosystem recovery. We hypothesized that (i) communities in new ponds would be less stable over years, (ii) species diversity is higher in reference sites than in new ponds for the whole community but not for active dispersers, (iii) communities in new ponds converge in their taxonomic composition with reference sites towards the final phase of annual succession within an inundation, and (iv) new ponds would contibute more to nestedness than reference sites. Although new ponds differed in abiotic conditions in respect to reference sites, we found that six to seven years after restoration invertebrate richness and diversity in new ponds matched the levels reached within reference sites, whilst invertebrate abundance was even higher. As succession progressed within an inundation, communities in new ponds were first dominated by branchiopods, then by flying colonists such as Chironomidae and Coleoptera and, finally, by halotolerant taxa such as the beetle *Ochthebius viridis fallaciosus*. However, communities of new and reference ponds frequently did not converge toward the end of inundations, but showed divergent community composition. Contrary to expectations, new ponds did not contribute more than reference ponds to the overall nested patterns, and contained taxa not found in reference sites, suggesting that, to date, they provide complementary habitats important for maintaining macroinvertebrate diversity.

Introduction

Wetlands are dynamic ecosystems that support unique biodiversity and provide important ecosystem services (MEA 2005; Ramsar Convention Secretariat, 2006). However, they are vulnerable to land use changes (Nicolet et al., 2004) whilst, dependence on rainfall, overflowing rivers or groundwater for their filling makes them vulnerable to environmental change. Since 1900, most of the world's wetland surface has been lost (Davidson, 2014), and human activities such as overexploitation, land conversion and introduction of alien species continue to cause wetland degradation and loss worldwide (MEA, 2005). On the other hand, wetland restoration projects have become common practice, aimed at re-establishing ecosystem functions and reversing biodiversity losses (Budelsky & Galatowitsch, 2000; Nakamura, Tockner & Amano, 2006; Palmer, 2009; Bullock et al., 2011).

Post-restoration monitoring is essential to evaluate the effectiveness of restoration projects and to allow an adaptive management approach, but in many restoration projects monitoring is absent or inadequate (Ruiz-Jaen & Aide, 2005). Where monitoring exists, the overall restoration success is typically evaluated based on similarities between the restored sites and reference sites (Seabloom & Hall, 2003; Moseman et al., 2004; Matthews & Spyreas, 2010; Meyer, Whiles & Baer, 2010). However, equally important is to compare the temporal dynamics of the restored versus the reference habitats and communities to visualize the stages of recovery over time. Community assembly typically involves deterministic processes that result in predictable changes in species composition controlled primarily by environmental gradients. However, stochastic processes such as long distance dispersal, priority effects and stochastic extinction can also modulate the outcome of species interactions over time (Chase, 2007). In a conservation context, this means that there are typically many potential routes to recovery after a disturbance, as well as a myriad of undesirable scenarios (Bond & Lake, 2003; Watts, Clarkson & Didham, 2008; Matthews & Spyreas, 2010; Suding, 2011; Brudvig, 2011). Temporal trajectories might thus help to identify the internal or external factors that cause change in community composition, both during inundations as well as long term changes across different years.

In general, new ponds are rapidly colonized by species from nearby water bodies (Williams, Heeg & Magnusson 2007), which may serve as reference sites. As a consequence, species assemblages in new ponds can be nested (i.e. impoverished) subsets of those in reference sites (Patterson, 1987), especially in the early years after wetland restoration (Ruhí et al., 2013). Information about nestededness patterns, and how these change over time, can thus be important to fully evaluate the potential value of restored sites for biodiversity conservation.

The present study is centred in the "Caracoles estate" within Doñana National Park (South West Spain), where a large complex of new ponds was created during marsh restoration. The Doñana marshes have been subject to strong anthropogenic impacts since 1920, including large-scale conversion of marshlands to cropland for intensive agriculture (Méndez et al., 2012). In 1998, the Spanish Ministry of Environment formulated a project to restore natural hydrology and reverse wetland loss. This allowed restoration of 5,600 ha of marshland, including the Caracoles estate (2,700 ha) which was incorporated into the National Park (Zorrilla-Miras et al., 2014; Sebastián-González & Green, 2014).

Macroinvertebrates play a critical role in wetland food webs by linking primary production to higher level consumers, such as birds and fish. They are also very useful as indicators of ecosystem processes, such as energy allocation and transformation (Covich, Palmer & Crowl, 1999; Balcombe et al., 2005) and ecosystem health (Sharma & Rawat, 2009; Florencio et al., 2009; 2011). They can indicate ecological perturbations that occur over time, including heavy metal accumulation, eutrophication or a modified flow regime (Feld & Hering, 2007). Monitoring macroinvertebrates provides very different insights compared to water birds, which are more frequently used in monitoring programs but which are less sensitive to ecological change (Guareschi et al., 2015).

Here we investigated the value of the Caracoles restoration project in supporting aquatic macroinvertebrates, six years after restoration. We compared species diversity and composition between newly constructed ponds and reference sites across two subsequent inundations in consecutive years. We compared patterns for the entire macroinvertebrate community with those for the speciose Coleoptera and Hemiptera. Macroinvertebrates have a broad range of dispersal abilities. The arrival of passive dispersers to new habitats can be slowed down by spatial constraints even if habitat conditions are suitable. In contrast, active dispersers such as adult Hemiptera and Coleoptera should be affected more by environmental control than by spatial processes (Heino, 2013). As a consequence, active dispersers may be better than the whole community for evaluating short-term ecosystem recovery. In addition, Coleoptera include particularly sensitive taxa (Bloechl et al., 2010; Van de Broeck et al., 2015), which may be good indicators of environmental differences between new and restored ponds.

The general aim of this study was to evaluate if wetland restoration allowed the development of macroinvertebrate assemblages similar to those in natural wetlands within 6 to 7 years. We tested the following specific hypotheses: (i) interannual variation in community structure would be more pronounced in new ponds due to their young age, in contrast to the older, mature reference sites; (ii) species diversity would be higher in reference sites than in new ponds for the whole community, but not for Coleoptera and Hemiptera because of their high dispersal capacity; (iii) communities in new ponds become more similar to reference sites towards the final phase of succession within an inundation, when environmental filters become stronger (e.g. temperature and salinity increase) and allow only the less sensitive taxa to persist; and (iv) when all sites are subject to a nestedness analysis, the ranks of new ponds would be higher in absolute value then those of reference sites, with a higher contribution for active dispersers. This is because new ponds are expected to provide a simpler environment, and to lack many passive dispersers owing to spatial constraints.

Material & Methods

Study area and climate description

The study was conducted within and around the Caracoles estate in the northern edge of Doñana National Park (South west Spain) (Fig. 1). This is a marshland area covering 2700 ha situated in the Guadalquivir estuary. During the 1960s, the estate was hydrologically disconnected from the surrounding marshes to prevent flooding and was converted into arable farmland. For over 30 years it was used mainly for the cultivation of cereals. During 2004 – 2005 restoration was carried out in the estate during the "Doñana 2005" government sponsored programme, with the aim of restoring its connection with surrounding marshes and creating a set of 96 experimental temporary ponds. These ponds were of similar elliptical shape but with variable long axis (60, 125 and 250 m) and maximum depth (30 and 60 cm). Most ponds were distributed in two clusters of 44 ponds each. In addition, 8 isolated ponds were constructed well away from these clusters (Supplementary Fig. 1). The colonization of these ponds by zooplankton and by water birds has been studied previously (Badosa et al., 2010; Frisch et al., 2012; Sebastián-González & Green, 2014).

Doñana has a Mediterranean climate with rainfall concentrated between October and March (wet season) and little precipitation from April to September (dry season). Caracoles ponds are usually flooded during the wet season and dry out from early May onwards until July. However, dates of flooding and drying vary among years, as a result of different rainfall and evaporation patterns. See Frisch et al. (2012) and Sebastian-Gonzalez & Green (2014) for more details of the study area.

Site selection and data collection

We sampled 32 new ponds within the Caracoles estate representative of all size and depth classes that were created, of which 24 were within the two clusters and 8 medium sized ponds were isolated and occurred outside these clusters (Supplementary Fig. 1). Most selected ponds had already been studied for zooplankton by Badosa et al. (2010). We also sampled ten older temporary reference sites representing different categories of water body types including three streams, two lakes, one artificial pond and one drainage ditch (see Fig. 1 and Supplementary Table 1 for details) that were located nearby. These sites have broader range of environmental variables than new ponds (Supplementary Table 1), but were selected to be both relatively accessible and close to the new ponds.

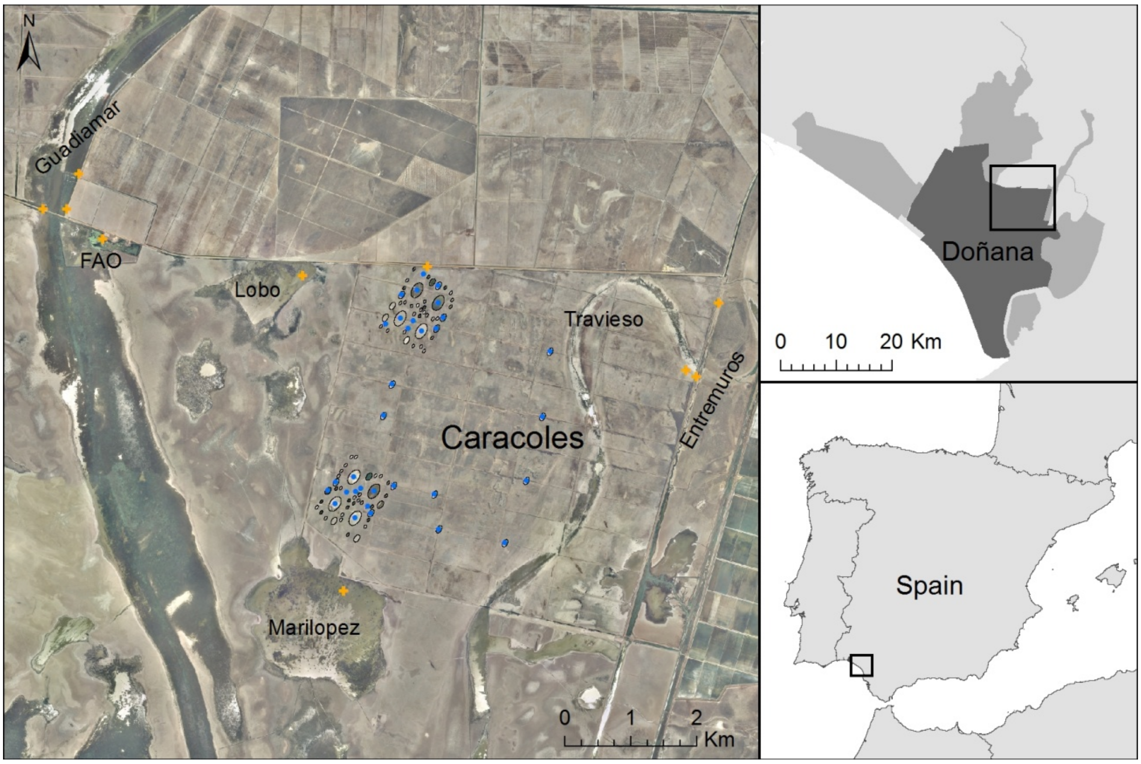

Figure 1. Map of the sampling sites in Doñana. The dark grey area in the upper figure indicates Doñana National Park and the light grey area indicates Doñana Natural Park. Blue points identify sampled new ponds within the Caracoles estate, orange crosses identify reference sites.

Sampling was conducted during two consecutive inundations (2009 - 2010 and 2010 – 2011 hereafter referred to as 2010 and as 2011) and started approximately 2 months after initial pond filling and was then repeated every 45 days, resulting in a total of 4 sampling events within each inundation before the ponds dried out (February, March, May and June). Total precipitation was 784 mm for the first inundation and 712 mm for the second inundation with much higher rainfall from December to February in the first (Supplementary Fig. 2). In both inundations, new ponds and reference sites showed similar timing of flooding and drying. The heavy floods that occurred especially during the first inundation established temporary connections between some ponds and surrounding reference sites (Supplementary Fig. 3). During the first inundation the connections between ponds in clusters and some reference sites (Supplementary Fig. 3) inhibited access to the other part of the estate, preventing complete sampling of the area (see Supplementary Table 2 for sampling details).

Environmental variables measured in ponds

During each visit we visually estimated the percentage of each pond area that was inundated, and water depth profiles were recorded with a measuring stick in five locations evenly distributed in the water body. We measured *in situ* the pH, salinity and temperature with a WTW 340i multiprobe. We also collected water samples for further analyses in the laboratory of chlorophyll-a, nutrient concentrations (total phosphorous and nitrogen) and turbidity. Chlorophyll *a* concentration ($\mu g\ l^{-1}$) was determined using methanol extraction (Talling & Driver, 1963). Total phosphorus was determined by colorimetry after acid hydrolysis (APHA, 1980). Total nitrogen was digested with alkaline potassium persulfate (D'Elia, Steudler, & Corwin, 1977) and absorbance measured at 220 nm. The presence of fish was determined by visual inspection of the net after sweeping, or in the laboratory during sample processing. The presence of submerged vegetation was recorded and total emergent vegetation cover (%) was estimated visually.

Sampling and processing of macroinvertebrates

In each pond on each sampling occasion, 3 samples of macroinvertebrates were collected using a D-framed pond net (500 µm mesh; 16 × 16 cm) by sweeping at 5 meter intervals (at 0, 5 and 10 m from the shore) along a transect. At each of the three points a 1-m sweep of 30s was carried out (Supplementary Fig. 4). Invertebrate samples were preserved in plastic containers filled with 70% ethanol. Data from the three samples were pooled for each pond and date before analysis.

Macroinvertebrates were identified in the laboratory under a stereo-microscope. The Coleoptera, Hemiptera and Crustacea were identified mostly to species level (after Jansson, 1986; Friday, 1988; Vondel, 1991; Alonso, 1996; Nieser et al., 1994); Odonata, Gastropoda and

Ephemeroptera to genus level (Carchini, 1983; Gerken & Sternberg, 1999; Tachet et al., 2000); and Diptera, Trichoptera and Lepidoptera to family level (Tachet et al., 2000). We also counted the Collembola, Hydracarina, Coelenterata, Turbellaria, Oligochaeta and Hirudinea, but did not identify them to a lower taxonomic level. For Coleoptera and Hemiptera, some juveniles could be identified only to genus level and were assigned to species according to the proportions of congeneric adults recorded in the same pond. If adults were not present, juveniles were left at genus level. Not all Hemiptera instars could be classified to genus, and these were left at family level.

Statistical analyses

All analyses were conducted in the statistical programming environment R version 2.15.3 (R Development Core Team, 2009), and functions in the packages Vegan (metaMDS, adonis, simper, nestednodf, oecosim, diversity, raupcrick, rarefy, speaccum), Bipartite (nestedrank) and Coin (Wilcox_test, permutational test).

Environmental variables

To investigate annual changes in abiotic conditions within new ponds and reference sites, and temporal differences between them, we used a Permutational Multivariate Analysis of Variance with distance matrices (PERMANOVA; “ADONIS” in R, see Oksanen et al., 2012). Analyses were conducted on log (x+1) transformed data (with the exception of pH and variables expressed as presence/absence). Annual dissimilarity among new ponds or reference sites and differences between them for each sampling occasion were calculated using Euclidean distances. To overcome the problem of differences in the number of sampled ponds between years (Supplementary Table 2), inter annual differences were tested using samples from May, as this was the month with the highest number of sampled sites each inundation. Differences between new and reference ponds were tested for each sampling occasion.

When ADONIS revealed significant differences in new ponds or in reference sites between years, or significant differences between reference sites and new ponds on a given sampling occasion, we performed a Multivariate Homogeneity of Group Dispersion (SIMPER) analysis. This analysis identifies the most influential explanatory variables for differences in environmental conditions between inundations, or between new ponds and reference sites. The significance levels were calculated using Wilcoxon paired tests (for temporal differences) and Mann-Whitney U tests, respectively.

Macroinvertebrate inter-annual abundance and diversity

Absolute richness and diversity were calculated using the lowest possible taxonomic level, generally genus and higher levels. Estimates of absolute richness were thus conservative. However, we used rarefied richness to compare between new and reference sites, which was performed using the minimum number of individuals collected within new ponds or reference sites between the two years. Annual changes in rarefied richness, the Shannon-Wiener diversity index and relative abundance (individuals/m^2) were performed separately for new and reference ponds using the data from May by means of Wilcoxon paired tests. Given the differences in sampling effort between inundations we also estimated richness using sample-based rarefaction curves based on the Chao 2 estimator.

Compositional similarity between inundations for new ponds or reference sites was tested using an ADONIS analysis on the abundance Hellinger transformed Euclidean distance matrix (i.e. Hellinger distances, see Legendre & Gallagher, 2001). Rare species that occurred in only one pond were excluded to avoid potential bias. If significant differences were found, we then performed a SIMPER analysis to identify the taxa that accounted for such differences. All analyses were done on the total community and for Coleoptera and Hemiptera separately.

Temporal dynamics in the community within inundations

We first visualized the differences in community structure between new ponds and reference sites for each month during each inundation using non-metric multidimensional scaling (NMDS) with the above mentioned distance matrices.

We then compared rarefied richness, which was standardized according to the minimum number of individuals recorded each month, Shannon-Wiener diversity indices and relative abundance (individuals/m^2) between new and reference ponds for each sampled month by means of a Mann-Whitney U test.

We also tested the similarity in community composition between new and reference ponds using a month-by-month ADONIS analysis based on Hellinger distances. SIMPER analysis was used to identify the taxa that contributed most to differences between new and reference ponds. Analyses were conducted on the whole community and on the Coleoptera and Hemiptera separately.

Finally, we investigated the compositional similarity between new and reference ponds controlling for the environmental variables that differed significantly between them each month with ADONIS. As the order of incorporation of non-orthogonal variables can impact the outcomes of significance testing in this procedure, the explanatory descriptors were introduced as the first predictors in the analysis, adding water body type as the last variable. This procedure allowed measurement of the pure effect of water body type after controlling for environmental variability, which was largely responsible for their differences.

Temporal trajectories in community assembly within inundations

To assess whether communities in new ponds become more similar to reference sites during the final phase of succession within an inundation we used a modified version of the Raup-Crick index (Chase et al., 2011), which is robust to variation in species richness. This index uses presence/absence data to express the probabilistic dissimilarity between two observed communities relative to the null expectation under a random assembly. The null expectation was generated using 9999 randomizations of a null model. This index was calculated using one single matrix containing data from both new ponds and reference sites.

We first tested for differences between the individual contributions to each pond type by means of Mann Whitney U tests on the averaged pairwise dissimilarity of all pairwise comparisons within the group. We tested if the dissimilarity index within each taxonomical group was affected by pond type (i.e., new ponds and reference sites). If pond type affected community composition, the pairwise dissimilarity indices between communities in new ponds and reference sites should be greater than those within new ponds. Because the pairwise dissimilarity indices calculated either among different new ponds or between new ponds and reference sites were not independent, the significance of this comparison was tested via Permutational Multivariate Analyses (ADONIS).

Temporal nestedness

To evaluate the contribution of new and reference ponds to nested patterns, we carried out nestedness analyses based on nested overlap and decreasing filling (NODF, see Almeida-Neto et el., 2008). NODF can assume values ranging from 0 to 100, with higher values indicating higher nestedness of communities. Nestedness analyses were calculated on presence/absence data, for a maximally nested matrix (i.e. sites ranked in decreasing order of species richness, and species ranked in decreasing order of incidence, so that the most diverse assemblage will occupy the first row and have a nested rank = 1). The significance of nestedness was evaluated by comparing observed values with those generated by 999 null models randomized according to a quasi swap algorithm (Gotelli & Entsminger, 2001). The quasi swap method creates independent matrices that maintains both row and column frequencies. This method is less vulnerable to Type I error (Gotelli, 2000). When significant nested patterns were detected, we tested for significant differences between the nested rank of reference and new ponds using a Mann-Whitney U test with 999 permutations.

Analyses were repeated for each sampled month, each inundation and for each taxonomic group separately.

Results

Environmental variables

New ponds and reference sites showed significantly different environmental conditions in the two study inundations (ADONIS, $P < 0.05$). Submerged vegetation, pH and turbidity explained ~50 % of cumulative differences between inundations for new ponds. During the second inundation, turbidity was significantly lower (Wilcoxon paired test, V = 454.5, $P < 0.001$), but pH was significantly higher (V = 35.5, $P < 0.001$) and submergent vegetation was detected in a higher number of ponds (Table 1). Submergent vegetation, pH and chlorophyll-a explained ~50 % of cumulative differences between inundations for reference sites. During the second inundation, pH and chlorophyll-a were not significantly higher but submergent vegetation was detected in a higher number of reference sites (Table 1).

There were significant differences between new ponds and reference sites (ADONIS, $P < 0.05$) each month, for each inundation. Mean monthly values are shown in Table 1. Dissimilarity between them was higher in June during both inundations (ADONIS, ~11%), when new ponds also exhibited significantly higher salinity (Mann Whitney U test, W_{2010}=60 $P = 0.003$; W_{2011}= 116, $P = 0.007$). Overall SIMPER analysis revealed that pH, chlorophyll-a content, vegetation (emergent and submergent), fish and turbidity together explained ~50 % of cumulative monthly dissimilarity between new ponds and reference sites, but they had different contributions each month (Supplementary Table 3). When differences in individual variables between new ponds and reference sites for a given month were significant, new ponds consistently had higher pH and presence of submerged vegetation, and lower chlorophyll-a concentration and presence of emergent vegetation and fish (detailed results not shown).

See Supplementary Table 4 for detailed information on vegetation and fish identified during this study.

Table 1. Environmental characteristics (±SE) of new ponds and reference sites over the study period. In the case of fish, vegetation (emergent and submergent) and *T. verticalis* (TV) the values indicate the number of sites where they were detected. Emergent vegetation refers to the presence of *Juncus subulatus* and *Scirpus maritimus*.

	2010				**2011**			
New Ponds	**Feb**	**Mar**	**May**	**Jun**	**Feb**	**Mar**	**May**	**Jun**
N	12	14	32	8	30	32	32	14
Mean depth	55.72	54.82	28.28	16.84	38.71	46.13	26.23	15.41
(cm)	(5.16)	(5.2)	(3.12)	(4.16)	(2.96)	(2.83)	(2.77)	(3.09)
Temperature	14.70	16.33	19.03	19.58	11.22	18.62	23.68	22.11
(ºC)	(0.51)	(0.61)	(0.68)	(1.57)	(0.31)	(0.57)	(0.57)	(0.84)
	8.29	8.44	9.03	8.98	8.91	9.23	9.43	9.07
pH	(0.03)	(0.12)	(0.05)	(0.13)	(0.03)	(0.08)	(0.08)	(0.08)
	0.79	0.36	2.14	5.88	0.76	0.68	2.32	13.06
Salinity (psu)	(0.21)	(0.01)	(0.19)	(0.85)	(0.08)	(0.07)	(0.15)	(1.96)
Turbidity	54.78	226.36	82.49	94.49	64.23	50.95	47.80	77.04
(NTU)	(9.58)	(21.95)	(10.38)	(24.80)	(10.06)	(5.61)	(5.84)	(15.61)
Total N	0.89	2.30	1.89	4.54	1.10	0.93	1.73	7.37
(mg l^{-1})	(0.08)	(0.21)	(0.16)	(0.78)	(0.11)	(0.08)	(0.12)	(1.10)
Total P	0.18	1.28	0.25	0.77	0.21	0.18	0.15	0.40
(mg l^{-1})	(0.02)	(0.77)	(0.04)	(0.11	(0.02)	(0.02)	(0.02)	(0.06)
Chla	2.95	9.27	18.88	74.34	5.72	3.33	8.5	42.15
(µg l^{-1})	(0.37)	(1.25)	(4.81)	(15.17)	(0.58)	(0.36)	(1.68)	(10.80)
Fish presence	0	3	4	2	1	9	7	9
Emergent								
Vegetation	0	1	25	4	5	28	31	13
Submerged								
vegetation	0	0	14	0	4	28	29	8
TV presence	0	0	7	5	3	5	13	11
Reference sites								
N	1	9	10	8	10	10	10	10
Mean depth		64.24	38.17	28.16	58.54	56.32	38.34	20.42
(cm)	61.17	(7.10)	(6.88)	(6.43)	(6.38)	(9.17)	(7.01)	(5.69)
Temperature		19.43	18.47	19.46	13.88	19.15	22.13	24.63
(ºC)	16.30	(0.47)	(0.85)	(1.26)	(0.50)	(0.87)	(0.67)	(0.93)
		8.25	8.45	8.29	8.46	8.64	8.47	7.97
pH	8.40	(0.14)	(0.13)	(0.18)	(0.04)	(0.23)	(0.19)	(0.17)
		0.21	1.33	2.15	0.52	0.53	1.58	5.27
Salinity (psu)	0.30	(0.08)	(0.37)	(0.49)	(0.07)	(0.08)	(0.22)	(1.28)
Turbidity		146.80	75.48	78.39	48.02	36.45	55.39	163.74
(NTU)	56.00	(32.61)	(10.99)	(16.53)	(7.72)	(13.10)	(13.76)	(44.92)
Total N		2.19	1.58	3.00	1.62	1.19	2.42	4.74
(mg l^{-1})	0.83	(0.18)	(0.35)	(0.47)	(0.30	(0.17)	(0.70)	(0.63)
Total P		0.34	0.27	0.42	0.18	0.19	0.33	0.61
(mg l^{-1})	0.26	(0.06)	(0.07)	(0.13)	(0.02)	(0.03)	(0.13)	(0.12)
Chla		11.51	15.98	37.81	15.02	9.12	15.66	72.91
(µg l^{-1})	3.52	(3.40)	(3.16)	(9.56)	(4.11)	(2.16)	(5.63)	(9.90)
Fish presence	0	1	6	4	1	5	7	9
Emergent	1	**9**	10	8	9	10	10	10
vegetation								
Submerged	0	0	2	3	3	7	8	3
vegetation								
TV presence	0	1	3	5	4	2	4	5

Macroinvertebrate abundance and diversity in different years

Over the two sampling periods we identified a total of 109 taxa (55 identified to species level belonging to 22 main taxonomical groups (Table 2). Insects dominated the fauna in new ponds and reference sites in both inundations. Coleoptera was the most taxonomically rich group with a total of 43 taxa, followed by Hemiptera with 17. Odonata and Diptera were represented by 9 and 14 taxa, although this was an underestimate since they were not classified beyond genus and family, respectively.

During the first inundation we found 73 taxa, of which 51 were recorded in new ponds, 66 in reference sites and 38 taxa were shared between new ponds and reference sites. During the second inundation in the next year we found 93 taxa, of which 74 were recorded in new ponds, 76 in reference sites, and 59 were shared. There were no significant differences between years in invertebrate abundance whether for new ponds or reference sites, for the whole community, Coleoptera or Hemiptera (Table 3). However, there was a significant increase in species richness and diversity in new ponds in the second inundation for all groups, whereas in reference sites we did not find any significant differences in species richness and diversity between years for each group (Table 3).

Community composition varied between inundations at the whole community level (ADONIS; $R^2 = 0.07$, $P = 0.001$) and for Coleoptera in new ponds ($R^2 = 0.05$, $P = 0.01$), and for Hemiptera in new ponds ($R^2 = 0.11$, P = 0.001) and reference sites ($R^2 = 0.14$, $P = 0.006$). The taxa identified from SIMPER analysis as discriminant among inundations and with the higher abundance in new ponds or in reference sites are reported in Supplementary Table 5.

Species accumulation curves showed an asymptotic trend for new and reference ponds for each group in each inundation, therefore any differences in sample size from 2010 to 2011 were unlikely to affect the above results (Supplementary Fig. 5).

Table 2. Total abundance and abbreviation (ID) of all taxa recorded in new ponds (P) and reference sites (R) during two consecutive inundations Numbers in brackets indicate the total number of sites where they were found.

			2010		**2011**	
Taxa	**ID**	**Family**	**P**	**R**	**P**	**R**
Collembola	Coll	-	0	0	9(4)	5(1)
Turbellaria	Turb	-	0	2(2)	0	0
Nematoda	Nema	-	2(1)	82(4)	0	0
Hydracarina	Acar	-	1(1)	0	2(2)	36(2)
Hydrozoa	Hydr	-	0	2(1)	0	0
Oligochaeta	Olig	-	14(4)	1014(6)	3(3)	806(9)
Hirudinea	Hiru	-	1(1)	0	0	0
Gastropoda		-				
Lymnaea spp.	Lym	Lymnaeidae	0	0	0	5(1)
Physa spp.	Phy	Physidae	0	152(7)	0	1351(7)
Planorbis spp.	Pla	Planorbidae	0	7(3)	0	10(2)
Gyraulus spp.	Gyr	Planorbidae	2(2)	101(5)	0	2842(5)
Ancylus fluviatilis Müll	Anc.fluv	Planorbidae	0	24(1)	0	178(4)
Anostraca						
Chirochephalus diaphanus Desmarest, 1823	Chi.diap	Chirocephalidae	94(12)	13(5)	98 (21)	19(3)
Streptocephalus torvicornis (Waga, 1948)	Str.torv	Streptocephalidae	217(18)	10(3)	3869(31)	37(4)
Notostraca						
Triops mauritanicus (Ghigi, 1921)	Tri.maur	Triopsidae	20(13)	14(6)	30(12)	3(2)
Spinicaudata						
Cyzicus grubei Simon, 1886	Cyz.grub	Cyzicidae	20(11)	13(3)	37(6)	31(3)
Brachipus spp.	Bra.sp	Branchipodidae	0	0	1(1)	0
Isopoda						
Asellidae spp.	Asell		0	0	0	1(1)
Amphipoda						
Corophium curvispinum Sars, 1895	Cor.curv	Corophiidae	0	1(1)	0	6(1)

Gammarus spp.	Gam	Gammaridae	0	1(1)	0	0
Decapoda						
Palaemonetes varians (Leach)	Pal.vari	Palaemonidae	0	1(1)	0	2(1)
Ephemeroptera						
Cloeon spp.	Clo	Baetidae	8(5)	115(7)	227(17)	34(8)
Ephemerella spp.	Eph	Ephemerellidae	0	0	1(1)	1(1)
Odonata						
Anax spp.	Ana	Aeshnidae	0	3(3)	1(1)	5(4)
Aeshna spp.	Aes	Aeshnidae	2(2)	4(1)	4(4)	30(8)
Aeshnidae spp.	Aesh	Aeshnidae	0	29(5)	18(1)	55(7)
Ischnura spp.	Isc	Coenagrionidae	11(6)	63(7)	129(16)	107(10)
Coenagrion spp.	Coe	Coenagrionidae	0	1(1)	7(4)	2(2)
Coenagrionidae spp.	Coen	Coenagrionidae	49(6)	143(9)	162(21)	281(10)
Gomphus spp.	Gom	Gomphidae	0	2(1)	0	0
Gomphidae spp	Gomp	Gomphidae	0	3(1)	0	0
Lestes spp.	Les	Lestidae	2(1)	12(4)	21(8)	88(6)
Lestidae spp.	Lest	Lestidae	0	2(1)	0	0
Crocothemis spp.	Croc	Libellulidae	0	1(1)	7(3)	6(3)
Orthetrum spp.	Ort	Libellulidae	0	0	5(4)	1(1)
Sympetrum spp.	Sym	Libellulidae	0	2(2)	12(7)	10(4)
Libellulidae spp.	Libe	Libellulidae	0	0	24(8)	13(4)
Trichoptera						
Hydroptilidae spp.	Hydr	Hydroptilidae	0	3(1)	0	0
Lepidoptera						
Crambidae spp.	Cram	Crambidae	6(4)	3(3)	1(1)	11(3)
Plecoptera		-	0	0	0	1(1)
Diptera						
Ceratopogonidae	Cera	Ceratopogonidae	9(4)	21(3)	2(1)	24(3)
Chironomidae	Chir	Chironomidae	14460(31)	8505(10)	23930(32)	9169(10)
Culicidae	Culi	Culicidae	0	65(1)	266(10)	28(5)

Dixidae	Dixi	Dixidae	0	0	0	6(2)
Dolichopodidae	Doli	Dolichopodidae	23(8)	0	2(2)	1(1)
Ephydridae	Ephy	Ephydridae	3(3)	10(1)	4(2)	396(5)
Limonidae	Limo	Limonidae	0	0	0	103(4)
Muscidae	Musc	Muscidae	54(4)	6(2)	0	0
Psychodidae	Psyc	Psychodidae	1(1)	1(1)	0	0
Rhagionidae	Rhag	Rhagionidae	5(2)	0	0	0
Sciomyzidae	Scio	Sciomyzidae	0	0	0	4(1)
Stratiomydae	Stra	Stratiomydae	1(1)	10(3)	0	10(4)
Tabanidae	Taba	Tabanidae	21(11)	21(7)	24(14)	19(8)
Tipulidae	Tipu	Tipulidae	0	0	3(2)	0
Diptera spp.	Dipt	-	5(2)	8(3)	4(3)	0
Coleoptera						
Bagous spp.	Bag.sp	Curculionidae	15(5)	21(4)	14(7)	5(3)
Curculionidae spp. (larvae)	Cur.sp	Curculionidae	0	0	7(4)	0
Dryops spp. (adults and larvae)	Dry.sp	Dryopidae	0	5(2)	0	10(4)
Agabus conspersus (Marsham 1802)	Aga.cons	Dytiscidae	2(2)	15(2)	8(4)	0
Agabus nebulosus (Forster, 1771)	Aga.nebu	Dytiscidae	0	0	3(1)	0
Agabus spp. (larvae)	Aga.sp	Dytiscidae	33(10)	10(1)	53(20)	19(4)
Colymbetes fuscus (Linnaeus, 1758)	Col.fusc	Dytiscidae	0	0	2(2)	0
Cybister spp. (larvae)	Cyb.sp	Dytiscidae	0	2(2)	1(1)	11(5)
Dytiscus sp. (larvae)	Dyt.sp	Dytiscidae	0	0	15(10)	4(2)
Graptodytes aequalis (Zimmermann, 1918)	Gra.aequ	Dytiscidae	0	0	0	1(1)
Graptodytes flavipes (Oliver, 1795)	Gra.flav	Dytiscidae	0	0	2(1)	0
Hydrovatus cuspidatus (Kunze, 1818)	Hyd.cusp	Dytiscidae	0	0	0	1(1)
Hydroglyphus geminus (Fabricius, 1792)	Hyd.gemi	Dytiscidae	7(4)	0	104(14)	57(6)
Hydaticus spp. (larvae)	Hyd.sp	Dytiscidae	1(1)	0	0	0
Hydroglyphus spp. (larvae)	Hydr.sp	Dytiscidae	2(2)	0	9(4)	4(2)
Hydroporus lucasi Reiche, 1866	Hyd.luca	Dytiscidae	0	0	7(3)	0
Hydroporus marginatus (Duftschmid, 1805)	Hyd.marg	Dytiscidae	0	0	3(1)	0

Hygrotus confluens (Fabricius, 1787)	Hyg.conf	Dytiscidae	98(11)	2(2)	188(20)	2(1)
Hygrotus lagari (Fery, 1992)	Hyg.laga	Dytiscidae	125(14)	10(1)	235(22)	176(1)
Hygrotus spp. (larvae)	Hyg.rosp	Dytiscidae	10(5)	7(2)	79(19)	27(3)
Hyphydrus spp. (larvae)	Hyp.drsp	Dytiscidae	0	0	0	1(1)
Ilybius spp. (larvae)	Ily.sp	Dytiscidae	2(1)	0	0	0
Laccophilus minutus (Linneaus, 1758)	Lac.minu	Dytiscidae	0	0	196(11)	7(1)
Laccophilus spp. (larvae)	Lac.sp	Dytiscidae	18(8)	99(2)	166(22)	233(7)
Rhantus (Rhantus) suturalis (McLeay, 1825)	Rha.sutu	Dytiscidae	0	0	2(2)	1(1)
Haliplus (Liaphlus) andalusicus Wehncke, 1874	Hal.anda	Haliplidae	56(12)	19(5)	154(22)	110(6)
Haliplus (Liaphlus) guttatus Aubé, 1836	Hal.gutt	Haliplidae	12(1)	3(1)	0	0
Haliplus spp. (larvae)	Hal.sp	Haliplidae	11(5)	0	79(13)	44(3)
Helophorus spp. (adults and larvae)	Hel.sp	Helophoridae	81(16)	80(6)	625(26)	51(6)
Ochthebius (Asiobates) dilatatus Stephen, 1829	Och.dila	Hydraenidae	0	8(3)	18(6)	4(2)
Ochthebius (Ochthebius) viridis fallaciosus Ganglbauer, 1901	Och.viri	Hydraenidae	847(21)	84(4)	1340(26)	57(7)
Ochthebius viridescens Ienistea, 1988	Och.vrds	Hydraenidae	0	0	1(1)	0
Ochthebius notabilis (Rosenhauer, 1856)	Och.nota	Hydraenidae	3(2)	0	0	0
Ochthebius (Ochthebius) punctatus Stephen, 1829	Och.punc	Hydraenidae	0	0	1(1)	0
Ochthebius spp. (larvae)	Och.sp	Hydraenidae	0	3(2)	0	0
Berosus (Berosus) affinis Brullé, 1835	Ber.affi	Hydrophilidae	1272(15)	434(5)	2664(28)	321(6)
Berosus (Enoplurus) guttalis Rey, 1883	Ber.gutt	Hydrophilidae	4801(27)	279(6)	6069(32)	517(10)
Berosus hispanicus Sharp, 1882	Ber.hisp	Hydrophilidae	0	27(3)	10(2)	52(5)
Berosus (Berosus) signaticollis (Charpentier, 1825)	Ber.sign	Hydrophilidae	0	2(1)	2(1)	42(5)
Berosus spp. (larvae)	Ber.sp	Hydrophilidae	178(8)	151(7)	109(9)	221(10)
Enochrus (Lumetus) bicolor (Fabricius, 1792)	Eno.bico	Hydrophilidae	0	0	40(12)	5(2)
Enochrus (Lumetus) fuscipennis (C.G. Thomsom, 1884)	Eno.fusc	Hydrophilidae	0	0	22(4)	16(2)
Enochrus halophilus (Bedel, 1878)	Eno.halo	Hydrophilidae	0	0	3(2)	4(1)
Enochrus spp. (larvae)	Eno.sp	Hydrophilidae	0	15(4)	37(11)	54(4)
Helochares (Helochares) lividus (Forster, 1771)	Hel.livi	Hydrophilidae	0	0	6(1)	2(1)
Helochares spp. (larvae)	Hel.chsp	Hydrophilidae	1(1)	2(1)	2(1)	6(2)

Hydrochara flavipes (Steven, 1808)	Hyd.flav	Hydrophilidae	0	0	1(1)	0
Hydrochara spp. (larvae)	Hyd.chsp	Hydrophilidae	0	3(1)	0	0
Hydrobius fuscipens (Linneaus, 1758)	Hyd.fusc	Hydrophilidae	1(1)	0	11(2)	23(1)
Hydrobius spp. (larvae)	Hyd.sp	Hydrophilidae	4(2)	17(1)	2(2)	24(4)
Gyrinus dejani Brullé, 1832	Gyr.deja	Gyrinidae	0	3(1)	61(3)	15(2)
Gyrinus spp. (larvae)	Gyr.sp	Gyrinidae	0	0	16(9)	6(2)
Hygrobia spp. (larvae)	Hyg.sp	Paelobiidae	0	0	5(1)	3(2)
Noterus laevis Sturm, 1834	Not.laev	Noteridae	0	0	0	17(4)
Augiles spp.*	Aug.sp	Heteroceridae	0	0	1(1)	3(1)
Heterocerus spp.*	Het.sp	Heteroceridae	0	0	1(1)	0
HEMIPTERA						
Corixa affinis Leach, 1817	Cor.affi	Corixidae	122(15)	45(5)	627(25)	88(7)
Corixa spp. (nymphs)	Cor.sp	Corixidae	14(7)	11(3)	85(17)	6(2)
Corixidae spp. (nymphs)	Cori.sp	Corixidae	7158(32)	1319(9)	6038(32)	1288(10)
Micronecta scholtzi (Fieber, 1860)	Mic.scho	Corixidae	0	52(4)	5(2)	227(6)
Micronecta spp. (nymphs)	Mic.sp	Corixidae	3(2)	192(2)	2(1)	13(5)
Paracorixa concinna (Fieber, 1848)	Par.conc	Corixidae	10(7)	0	0	0
Sigara (Vermicorixa) lateralis (Leach, 1817)	Sig.late	Corixidae	1695(28)	1061(8)	2426(31)	186(10)
Sigara (Vermicorixa) scripta (Rambur, 1840)	Sig.scri	Corixidae	67(5)	98(4)	404(18)	76(3)
Sigara (Halicorixa) stagnalis (Leach, 1817)	Sig.stag	Corixidae	21(5)	21(3)	221(15)	6(1)
Sigara spp. (nymphs)	Sig.sp	Corixidae	10(4)	5(2)	18(7)	22(4)
Trichocorixa verticalis (Fieber, 1851)	Tri.vert	Corixidae	154(9)	178(6)	540(23)	125(10)
Anisops sardeus Herrich-Schäffer, 1849	Ani.sard	Notonectidae	40(12)	65(3)	278(20)	30(2)
Anisops spp. (nymphs)	Ani.sp	Notonectidae	28(6)	9(2)	24(11)	9(4)
Notonecta glauca Linnaeus, 1758 ssp. *glauca*	Not.glgl	Notonectidae	0	0	2(2)	0
Notonecta glauca Linnaeus, 1758 ssp. *meridionalis* Poisson, 1926	Not.glme	Notonectidae	0	1(1)	3(2)	0
Notonecta maculata Fabricius, 1794	Not.macu	Notonectidae	0	1(1)	0	0
Notonecta viridis Delcourt, 1909	Not.viri	Notonectidae	0	1(1)	13(4)	0
Notonecta spp. (nymphs)	Not.sp	Notonectidae	1(1)	0	3(2)	1(1)
Notonectidae spp. (nymphs)	Noto.sp	Notonectidae	309(23)	399(6)	374(25)	52(8)

Gerris brasili Poisson, 1940	Ger.bras	Gerridae	0	0	1(1)	0
Gerris (Gerris) thoracicus Schummel, 1832	Ger.thor	Gerridae	0	8(1)	14(4)	22(1)
Gerris spp. (nymphs)	Ger.sp	Gerridae	0	0	6(2)	1(1)
Microvelia pygmaea (Dufour, 1833)	Mic.pygm		1(1)	0	9(5)	3(1)
Plea minutissima Leach, 1817	Ple.minu	Pleidae	1(1)	0	167(3)	12(3)
Velia spp.	Vel.sp	Velidae	0	0	0	1(1)

* semiaquatic

Table 3. Mean (±SE) cumulative abundance (A; individuals/m^2), absolute richness (AR), rarefied richness (RR) and Shannon diversity (D) in new ponds (32) and reference sites (10) for the whole macroinvertebrate community and for Coleoptera and Hemiptera separately during May within each inundation. Asterisks indicate significant differences between inundations, according to Wilcoxon paired–tests, and are placed alongside the higher values.

	A	**AR**	**RR**	**D**	**A**	**AR**	**RR**	**D**
	2010				**2011**			
Total Macroinvertebrates								
New	1129.8	9.4	5.69	3.8	1096.9	14.4	7.99***	6.1***
Ponds	(214.7)	(0.5)	(0.33)	(0.2)	(120.8)	(1.0)	(0.49)	(0.5)
Reference	1600.1	12.9	6.27	4.1	1497.3	15.7	7.75	5.2
sites	(660.9)	(2.5)	(0.61)	(0.63)	(491.7)	(2.5)	(0.88)	(0.9)
Coleoptera								
New	368.5	3.6	2.79	1.7	445.8	6.9	4.93***	3.2***
Ponds	(63.3)	(0.3)	(0.25)	(0.1)	(66.5)	(0.7)	(0.46)	(0.3)
Reference	190.0	3.7	2.75	1.8	204.2	5	4.32	2.8
sites	(100)	(1.4)	(0.53)	(0.4)	(131.2)	(1.2)	(0.84)	(0.6)
Hemiptera								
New	420.0	3.1	2.88	2. 1	234.6	4.2	3.89***	3.2***
Ponds	(110)	(0.2)	(0.20)	(0.1)	(34.6)	(0.29)	(0.24)	(0.2)
Reference	297.0	3.2	3.02	2.5	224.9	2.9	2.83	2.2
sites	(91.9)	(0.7)	(0.43)	(0.4)	(155.4)	(0.2)	(0.42)	(0.2)

*$P < 0.05$
**$P < 0.01$
***$P < 0.001$

Comparing new pond and reference communities within inundations

The NMDS ordination showed different level of separations between new and reference ponds depending on the taxonomic group, the studied month and the inundation period (Fig. 2). We did not detect any significant differences in the whole community abundance between reference sites and new ponds each month, for either inundation, but richness and diversity were significantly lower in new ponds during February 2011 (Table 4). With the exception of June 2010, community composition differed between new ponds and reference sites each month for both inundatins (ADONIS; $P < 0.05$; see Supplementary Table 6 for the main differences in taxa). Coleoptera abundance was higher in new ponds than reference sites in May and June each inundation but rarefied richness and diversity were never significantly different between pond types (Table 4). Coleoptera composition differed between them almost every month (ADONIS; $P < 0.05$), except during March 2011 (see Supplementary Table 7 for the main differences in taxa). Hemiptera had higher abundance in new ponds than reference sites in June 2011 and higher rarefied richness in May 2011 (Table 4). Hemiptera composition did not differ significantly between new and reference ponds during the first flooding (ADONIS; *P* > 0.05), but it differed significantly each month during the second one (ADONIS; *P* < 0.05; see Supplementary Table 8 for the main differences in taxa).

Most of the variation in community composition between new ponds and reference sites was related to their environmental differences, and most differences among new ponds and reference sites disappeared after adjusting for environmental differences (cut off of cumulative percentage of dissimilarity applied at ~70%). However, differences between water body types remained significant for the whole community in March 2011 (ADONIS; $R^2 = 0.04$, $P = 0.032$), for Coleoptera in June 2010 ($R^2 = 0.15$, $P = 0.041$) and for Hemiptera in February 2011 ($R^2 = 0.07$, $P = 0.012$).

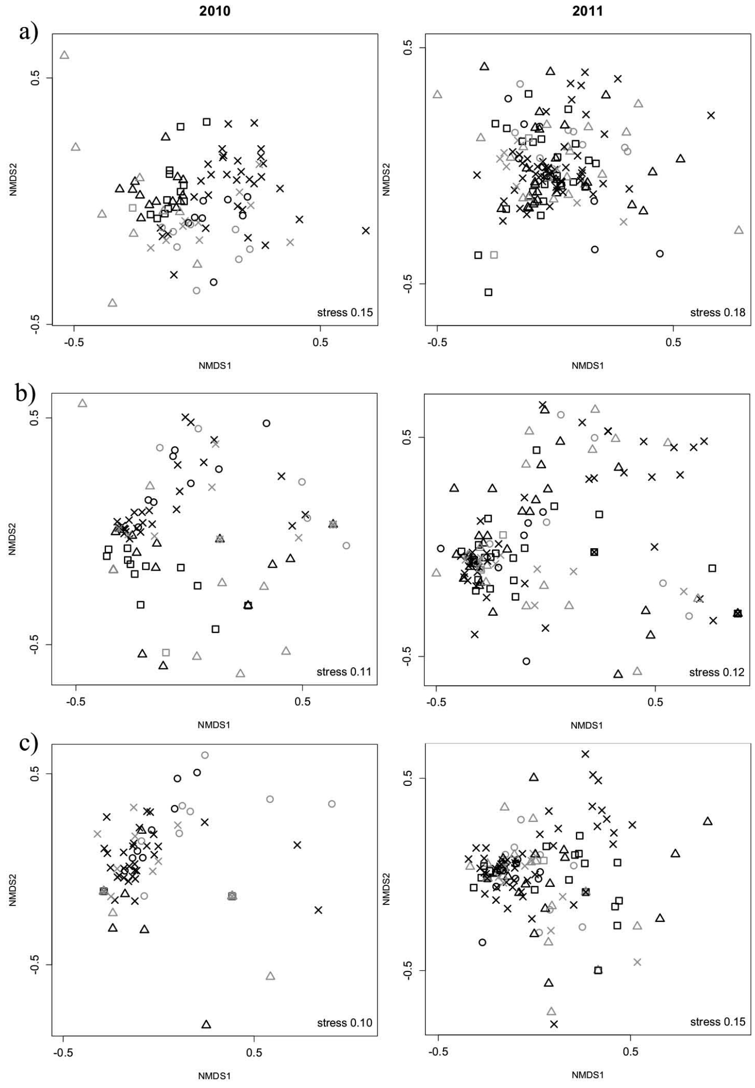

Figure 2. NMDS ordination of (a) the whole community, (b) Coleoptera and (c) Hemiptera abundance showing the differences in community composition between new ponds (black symbols) and reference sites (grey symbols) across months. Feb = □, Mar = △, May = x, June = ○.

Table 4. Mean (± SE) monthly abundance (individuals/m^2), absolute richness, rarefied richness and Shannon–Wiener diversity in new ponds and reference sites for the total macroinvertebrate community (M) and for Coleoptera (C) and Hemiptera (H) separately. TV = *Trichocorixa verticalis*. Asterisks indicate significant differences between reference sites and new ponds, and are placed alongside the higher values.

	2010				2011			
	Feb	**Mar**	**May**	**Jun**	**Feb**	**Mar**	**May**	**Jun**
N	12	14	32	8	30	32	32	14
				Abundance				
New Ponds								
M	255.20	229.46	1129.77	3092.34	620.13	1087.16	1096.87	1496.39
C	37.32	22.61	368.55*	404.16**	39.09	216.01	445.76*	244.78*
H	2.08	6.24	420.00	814.73	64.99	202.66	234.64	536.72**
TV	0	0	8.13	7.55	0.27	0.32	12.36	50.74
Reference sites								
N	1	9	10	8	10	10	10	10
M	/	165.73	1600.12	1758.02	404.78	1444.15	1497.27	832.76
C	/	16.97	190.00	77.34	52.70	104.78	204.16	87.28
H	/	6.24	297.00	524.82	65.83	77.91	224.99	82.77
TV	/	0.23	19.11	22.13	2.77	1.66	11.24	10.41
				Absolute Richness				
New Ponds								
M	7.8	6.9	9.43	10.35	8.6	10.	14.40	12.07
C	4	2	3.6	3.85	3.46	4.6	6.9	4.6
H	0.5	0.85	3.06	4.1	1.16	2.44	4.21	4.93
Reference sites								
M	/	9.77	12.9	11.25	13.3	15.7	15.7	8.6
C	/	2.4	3.7	2.87	5	5	5	3
H	/	0.66	3.2	3.5	1.8	2.6	2.9	2.6
				Rarefied Richness				
New Ponds								
M		3.08	5.8	4.57	3.44	4.10	7.99*	3.33
C		1.91	2.83	3.06	3.23	2.9	4.93	2.23
H		0.85	2.89	3.22	1.2	2.32	3.89	2.58
Reference sites								
M		4.58	6.41	5.16	6.56***	4.73	7.75	2.66
C		2.25	3.14	2.51	4.24	3.34	4.32	1.80
H		0.66	3.02	2.81	1.8	2.47	2.83	1.94
				Diversity				
New Ponds								
M	2.66	3.05	3.79	3.15	2.62	3.47	6.12	5.18
C	2.91	1.66	1.71	1.91	2.26	1.86	3.19	2.50
H	1	1.24	2.07	2.20	1.18	1.96	3.2	3.28
Reference sites								
M	/	5.06	4.10	3.51	5.50***	3.53	5.20	3.52
C	/	2.05	1.76	1.93	3.49	3.49	2.78	1.84
H	/	1.09	2.49	2.15	1.64	2.21	2.2	2.45

*P < 0.05
**P < 0.01
***P < 0.001

Temporal trajectories in community assembly within inundations

For the whole community the modified Raup-Crick dissimilarity index (β_{RC}) was significantly lower in new ponds compared to reference sites each month (i.e. comparing PP and RR in Fig. 3), during both inundations (Mann-Whitney U test, $P < 0.05$). Differences were not significant in March 2010 for Coleoptera (W = 32.5; $P = 0.06$) and Hemiptera (W = 62.5; $P = 1$) or in June 2010 for Hemiptera (W = 14; $P = 0.06$).

When comparing differences within new ponds with those between new ponds and reference sites (i.e. PP and PR in Fig. 3), the β_{RC} index was affected by pond type every month for the whole community and for months other than March for Coleoptera (ADONIS; $P < 0.05$). For Hemiptera the β_{RC} was affected by pond type in May 2010 ($R^2 = 0.05$, $P = 0.05$) and all months in 2011 except March (ADONIS, $P < 0.05$).

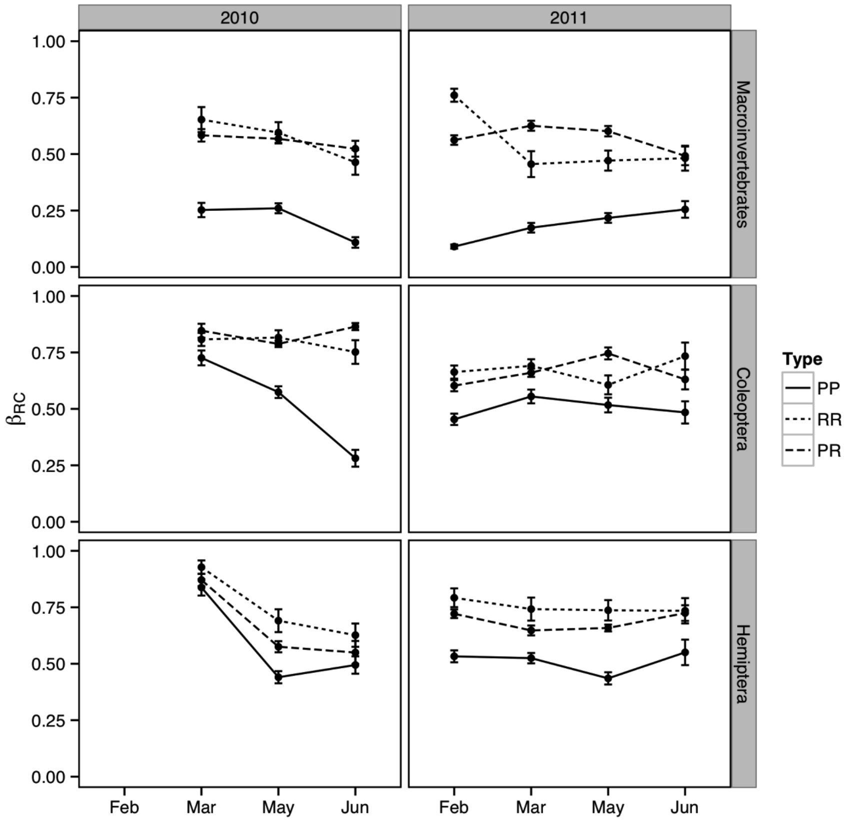

Figure 3. Mean pairwise Raup Crick dissimilarity indices for the whole community (Macroinvertebrates), Coleoptera and Hemiptera, within new ponds (PP), within reference sites (RR) and between them (PR). February 2010 data are missing because extensive flooding prevented the complete sampling of the area (Supplementary Fig. 3).

Temporal nestedness

During the first inundation, the overall macroinvertebrate community structure was never significantly nested, whereas Coleoptera were nested in May (NODF= 17.79, *P* = 0.08) and Hemiptera in June (NODF = 25.75, *P* = 0.04). During the second inundation, the whole community was significantly nested in March (NODF = 15.43, *P* =0.02), Coleoptera were nested in March (NODF = 21.53, *P* = 0.03) and June (NODF = 12.21, *P* = 0.017), whereas Hemiptera were nested in May (NODF = 44.56, *P* = 0.021) and June (NODF = 31.64, *P* = 0.019). In no case did new ponds have a significantly higher nestedness rank than reference sites.

Discussion

In this study we found that 6-7 years after restoration new ponds provided different and more homogeneous environmental conditions to reference sites but still supported diverse communities representative of natural systems and seem to be a well-integrated component of the Doñana wetland complex. Reference sites did not appear to act as a source of colonist for the newly created ponds within an inundation any more than new ponds acted as a source for reference sites.

Patterns of communities change over years

Over the two studied consecutive inundations, richness and diversity increased in new ponds for all invertebrate groups, whereas they were almost stable in reference sites. This result supports our first prediction that communities in new ponds are less stable over years than those in reference sites. The colonisation of new ponds occurs when passive dispersers are transported via vectors, or when actively flying insects (e.g. Coleoptera and Hemiptera) arrive from nearby water bodies (Williams, Heeg & Magnusson, 2007). Passive dispersers with dormant propagules (e.g. branchiopods or turbellarians; see Brendonck & De Meester, 2003) can recolonise temporary ponds in subsequent years when they are re-flooded. Hence one reason why richness increased in our second study year could be the increase in diversity of the propagule bank in the sediments. Newly created habitats have unoccupied niches that become rapidly colonized by agile, generalist taxa. As communities develop, more niches become available (e.g. through development of vegetation) and species with specialized requirements can progressively colonize. Particularly, development of vegetation is likely to be important in our system since it has been shown to be a prime driver of macroinvertebrate diversity in freshwater systems (Declerck et al., 2005). This is supported by the accumulation, or increased abundance, of taxa typically associated with macrophytes (e.g. *Laccophilus minutus*; *Enochrus* spp., Libellulidae, *Berosus affinis*). However, the difference between study years may also have been a consequence of the dynamic hydrology. Reduced connectivity and increased isolation between new ponds in the second year (Supplementary Fig. 3) could in itself be the cause of increases in beta and gamma diversity. Different communities can develop in similar but disconnected ponds because passive dispersers can largely be regulated by stochastic processes and priority effects (Scheffer et al., 2006). On the other hand, communities in reference sites seem to respond differently to variation in connectivity across inundations. This probably reflects their great habitat heterogeneity that maintains diversity within communities (Fig. 3).

Macroinvertebrate recovery and dispersal limitation

At a given point in time within each inundation, new ponds and reference sites often differed in community composition, richness and diversity. One major reason for this is that some taxa had not yet managed to colonize new ponds. The absence of some taxa (e.g. Gastropoda) from new ponds and the presence of others in low abundance (e.g. Oligochaeta) suggest that populations of these species have not yet (fully) established. Both taxa lack planktonic dispersal stages and Oligochaeta possess juveniles that develop in cocoons. These factors delay colonization of new habitats by Gastropoda and Oligochaeta, making their recovery very slow (Brady et al., 2002). These results support our second prediction that dispersal limitation will be more apparent for the whole community than for Coleoptera and Hemiptera with high flight ability.

On several occasions, new ponds and reference sites differed in community composition but not in richness and diversity, suggesting that some taxa showed site-specific preferences owing to variation in environmental conditions, as observed at intermediate phases of succession within an inundation. New ponds were usually fishless, a factor that may explain the dominance of the large branchiopods *Streptocephalus torvicornis* and *Chirocephalus diaphanus* that hatch from dormant egg banks. Hatching experiments suggest that these taxa were absent from the sediments when new ponds were created (Frisch & Green, 2007), suggesting that their eggs were rapidly dispersed either through water connections between new and reference ponds, or by the abundant water birds (Green & Figuerola, 2005; Sebastián-González & Green, 2014). The dominance of chironomids in the new ponds was to be expected (Layton & Voshell, 1991) and as with the large branchiopods, this might reflect their lack of dependence on higher vegetation. In contrast, among Coleoptera many Hydrophilidae are commonly associated with well-vegetated ponds with high pH (Bloechl et al., 2010; Touaylia, Garrido, & Boum aiza, 2013). New ponds generally had a higher coverage of submergent vegetation than reference sites. This difference could partly explain the higher abundances of adult *Berosus* (Hydrophilidae), found in new ponds in the later stage of an inundation. We cannot exclude, however, that lower predation pressure exerted by fish in new ponds also played a role. On the other hand, the lower abundance of Odonata reflects the lack of emergent vegetation in new ponds. As seasons proceed and temporary ponds begin to dry out, salinity increases causing osmotic stress to macroinvertebrates. In this advanced phase, only the most tolerant species could persist in the most saline new ponds, such as the Coleoptera *Ochthebius viridis fallaciosus* (Garrido & Munilla, 2008; Millán et al., 2011) or the Hemiptera *Sigara stagnalis* and the alien *Trichocorixa verticalis* (Van de Meutter, Trekels, & Green, 2010). However, our finding of higher Hemiptera richness in new ponds also reflects their acquisition of some widespread taxa absent from reference sites (e.g. *Notonecta glauca*).

Our finding that richness and diversity in new ponds matched the levels in reference sites 6-7 years after restoration, and that abundance even surpassed the reference levels, is not unusual in restored wetlands. Several studies have shown a rapid recovery of species richness and diversity in macroinvertebrates within a few years after wetland restoration (Meyer & Whiles, 2008), and this was previously observed for zooplankton in our new ponds (Badosa et al., 2010). In contrast, the recovery of natural community composition generally takes more time (Levin & Talley, 2002). However, we recorded some taxa exclusive to both pond types and nearly 40 Coleoptera taxa in new ponds (Table 2), including specialist herbivores (e.g. Curculionidae), indicating that the new ponds are healthy ecosystems of conservation value (Bameul, 1994).

Community similarity and the effect of pond type

Overall, new ponds supported communities that were more similar among them (including ponds within and outside the clusters) than for reference sites. This probably reflects greater habitat heterogeneity between individual reference sites, which include a broader range of waterbody shape, depth and other characteristics (Fig. 1; Supplementary Table 1). The slow recovery for emergent plants in new ponds, might have increased this difference between new and reference ponds. Emergent plants appear to be relatively poor at colonizing by seeds compared to submerged ones, and were mainly expanding their cover slowly by clonal horizontal expansion. Our results were similar to the results of a recent meta analysis (Moreno-Mateos et al., 2012), showing slow recovery rates for vegetation in restored wetlands.

Our results did not support our hypothesis that community composition between new and reference ponds becomes more similar towards the end of an inundation, when environmental conditions associated with desiccation may filter out sensitive taxa. In fact, the environmental dissimilarity between new ponds and reference sites increased towards the end of the hydroperiods, when the smaller new ponds became more saline through evaporation. This indicates that communities were shaped mainly by environmental differences between pond types, which were sufficiently strong to prevent convergence in community composition. Differences between pond classes in levels of dissimilarity within each class (comparing PP with RR in Fig. 3) were weakest early on in March, and became weak again later in June in the case of the Hemiptera, probably because of their broader niches (Bloechl et al., 2010). Typically, early colonists are the most generalist, and should therefore be the least affected by specific habitat properties (Vanschoenwinkel et al., 2010). The lack of differences between pond classes in March (comparing PP with PR in Fig. 3) suggest that pioneer taxa colonized indiscriminately both new and reference ponds, irrespective of their environmental differences. This suggests that deterministic processes were of less importance for early colonists. However, landscape structure may influence species dispersal (Numa et al., 2009) and during the first inundation, when our system was completely flooded (Supplementary Fig. 3), high turnover of Coleoptera and Hemiptera across pond types may have decreased the inter-pond class variation.

Differences in the abundance of invasive species between body types and between inundations may also have had strong effects. This concerns invasive fish (Supplementary Table 4) but also the alien corixid *T. verticalis* whose impact on other macroinvertebrates is unknown and is worthy of detailed investigation (Van de Meutter, Trekel & Green, 2010; Coccia et al., 2013). Within its native area *T. verticalis* exerted a top-down control on a tri-trophic food web (phyoplankton-cladoceran zooplankton-*T. verticalis,* see Simonis, 2013). In our study area, we found a clear negative correlation between the abundance of *T. verticalis* and that of grazers and periphyton consumers such as *Ochthebius viridis fallaciosus, Cyzicus grubei,* and Corixidae nymphs or Coleoptera larvae (unpublished results). This suggests that this invasive species can affect the community structures indirectly by competition for food more than directly by predation (e.g. on chironomids or cladocerans), and that *T. verticalis* might potentially exert bottom up control in these ponds. However, Cladocera are rare or absent in many of these ponds (Badosa et al., 2010; Frisch et al., 2012). Further studies are needed to clarify this point.

Contribution to global nestedness

A nested species assemblage occurs when taxa in sites with lower species richness are a proper subset of those in richer sites (Patterson, 1987). Nestedness is the result of a non random distribution of species between sites that differ in e.g., area, isolation or habitat diversity (i.e., nested habitats) (Patterson & Brown, 1991; Wright et al., 1998). Our initial hypothesis was that the more complex and mature reference sites would also contain more habitat specialists, and that new pond communities should be ranked higher in the maximally nested matrices than reference sites. We found no support for this hypothesis.

Overall, the communities in our system exhibited significant nested patterns across inundations. Previous studies have found macroinvertebrates to be highly nested in freshwater habitats (Ruhí et al., 2013), including a study in a different part of Doñana (Florencio et al., 2011). There are several factors that may have contributed to generate the nested species assemblage in our system, including differences in pond size or isolation (Kadmon, 1995; Heino, Mykrä & Rintala, 2010), whose effects may have been variable within and between inundations. However, whatever the cause of nested patterns we did not find any significant differences in the nested ranks between new and reference ponds during each inundation for any taxonomic group. Nestedness is a type of hierarchical organization of species, sites or both (Patterson & Atmar, 2000), so that the rank order of site reflects the suitability gradient among them (Azeria & Kolasa, 2008). In our maximally packed matrix, similar mean ranking values between new and reference ponds suggest that new ponds, despite being less heterogeneous than reference sites, are now well integrated into our wetland complex. Furthermore, it seems likely that their ecological attributes increased the overall habitat complexity of the area. This in turn suggests that reference sites didn’t act as a source of colonists for new ponds any more than new ponds were a source for reference sites, underlining the value of the new ponds for biodiversity conservation 6-7 years after restoration.

Conclusion

Our study shows that 6-7 years after restoration new ponds constructed in Doñana National Park (SW Spain), one of the most important wetland areas in Europe, hold different macroinvertebrate communities than natural reference sites. This may indicate that new ponds are still supporting pioneer communities. A longer time is usually needed to reach a stable, mature macroinvertebrate community assemblage in restored wetlands (Moreno-Mateos et al., 2012), which seems likely in our system given the slow recovery of emergent vegetation. However, communities in new ponds will not necessarily converge towards the composition of reference communities. Environmental factors seem to be the main drivers of community assembly in this area, and it seems likely that the environmental heterogeneity between new ponds and reference sites still remains sufficiently strong for macroinvertebrates to perceive new ponds as different habitats. Further studies will be needed to address this in the future. The success of restoration is often evaluated based on similarities in species composition, diversity and density between the restored sites and target reference sites. However, this study illustrates the need to consider patterns of temporal composition, including species identity, and aspects of local and regional processes to fully restoration success. Although different to reference sites, these newly created ponds are healthy ecosystems with considerable conservation value.

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Supplementary Materials

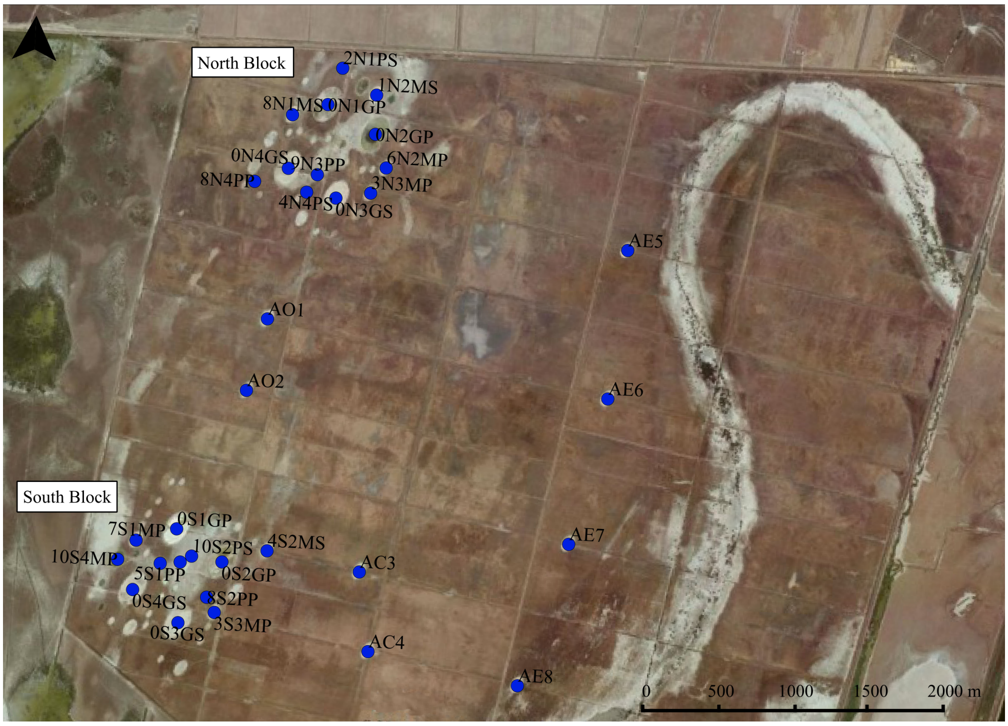

Figure 1. Codes of the sampled ponds within Caracoles estate

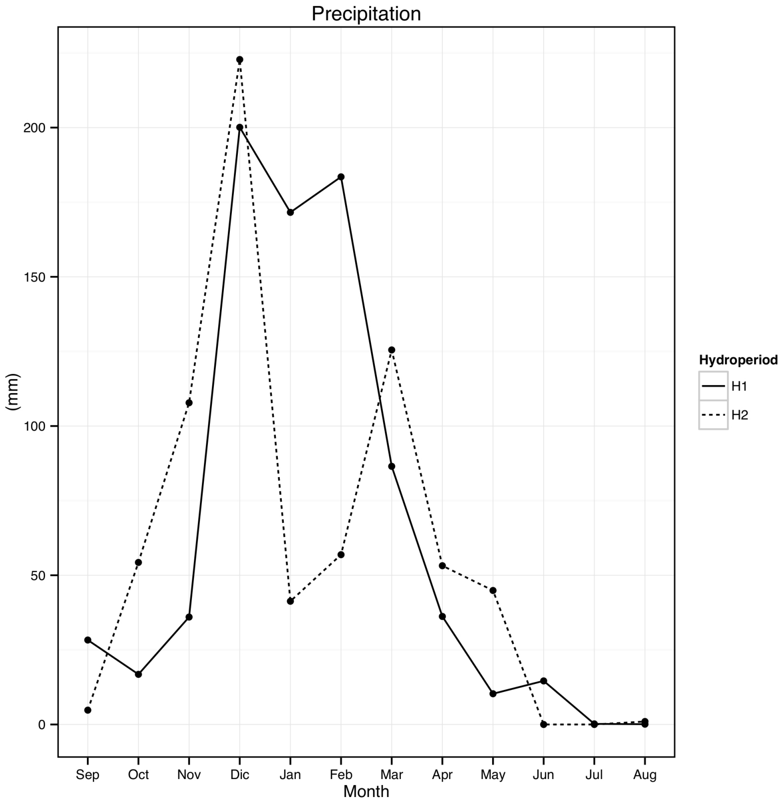

Figure 2. Monthly variation in precipitation (mm) during the two studied inundations (H1 = 2009 - 2010, total = 784 mm; H2 = 2010 - 2011, total = 712 mm).

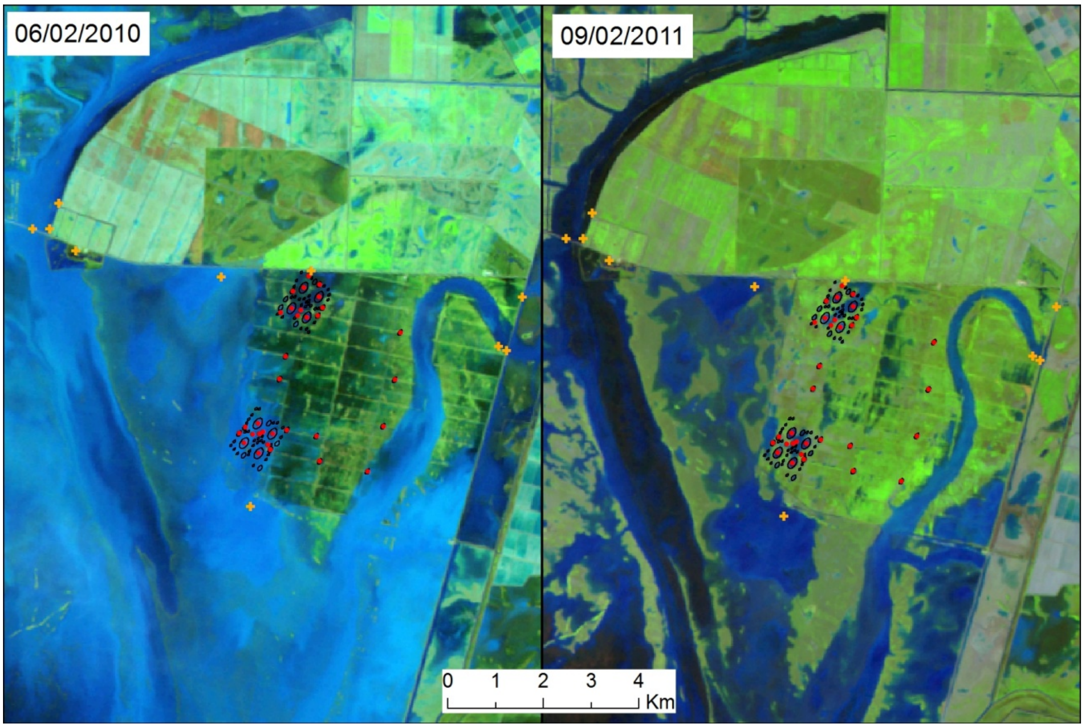

Figure 3. Landsat 7 ETM+ images showing the differences during peak flooding among inundations in the water surface area and the level of connectivity within the study area. Red points identify new ponds within Caracoles, orange crosses identify reference sites.

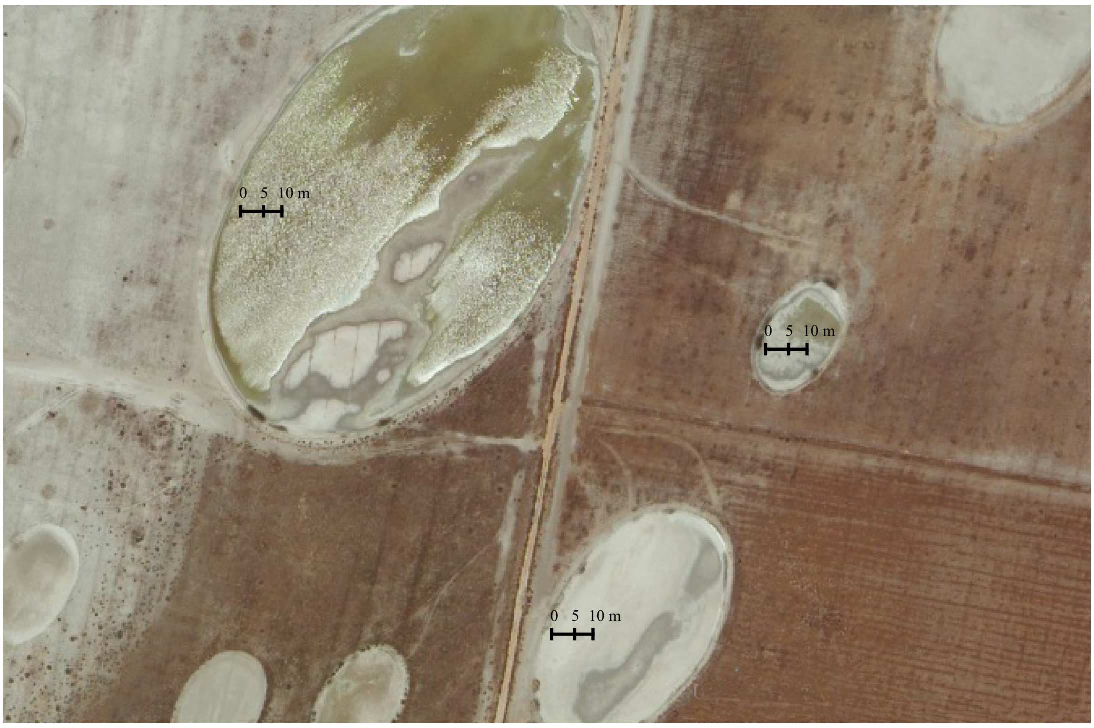

Figure 4. Examples of 10 m line transect within large, medium and little ponds. Biological samples were collected at 0, 5 and 10 m from the shore by sweeping a D-framed pond net (500 µm mesh; 16 × 16 cm) along 1 meter for 30s. Image from Google Earth.

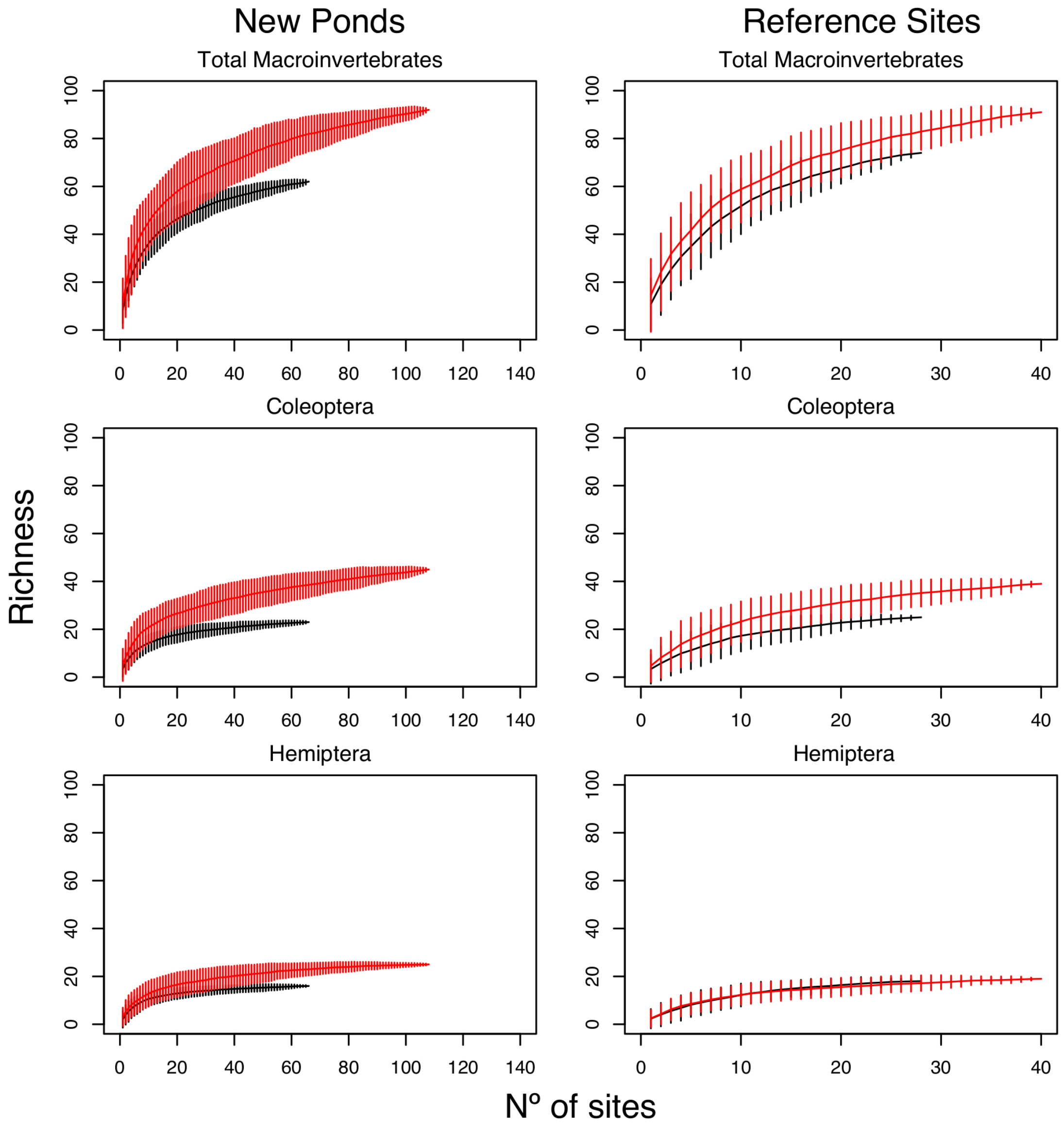

Figure 5. Rarefaction curves and standard error bars showing differences in estimated taxon richness between new ponds (black) and reference sites (red) for the whole community (total macroinvertebrates), Coleoptera and Hemiptera.

Table 1. Details of the environmental characteristics (range) of the ten reference sites.

	Caracoles channel	**Caño-1**	**Marilopez**	**Entremuros _1**	**Entremuros _2**	**El Lobo**	**Fao**	**Guadiamar _1**	**Guadiamar _2**	**Guadiamar _3**
	Drainage ditch	Seasonal temporary stream	Shallow temporary lake (300 ha)	Seasonal temporary stream	Seasonal temporary stream	Shallow temporary lake (120 ha)	Shallow, artificial pond (50 ha)	Seasonal temporary stream	Seasonal temporary stream	Seasonal temporary stream
2010										
Mean depth (cm)	15.0 - 61.2	9.9 - 62.4	39.2	10.9 - 39.0	5.3 - 35.0	14.9 - 71.2	32.4 - 52.2	58.2 - 85.4	46.0 - 92.6	19.0 - 87.8
Temperature (ºC)	16.3 - 22.7	14.4 - 17.8	22.0	16.1- 21.5	17.3 - 19.2	15.5 - 20.7	21.1 - 21.4	18.1 - 21.8	18.4 - 22.3	17.2 - 20.6
pH	7.9 - 8.5	8.8 - 9.3	8.46	8.3 - 8.6	8.2 - 8.3	8.1 - 8.8	7.9 -9.0	7.8 - 7.9	7.6 - 8.0	7.7 - 8.3
Salinity (psu)	0.3 - 3.6	0.4 -4.6	0.7	0.2 - 2.7	0.2 -1.7	0.1 - 2.2	0.7 - 4.2	0.0 - 0.9	0.0 - 1.0	0.0 - 1.0
Turbidity (NTU)	25.3 - 196	33.7 - 225	87.0	18.8 - 115	43.2 - 61.0	54.0 - 263.0	2.1 - 46.0	63.0 - 172.0	58.0 - 207.0	134.0 - 194.0
Total N (mg l^{-1})	0.8 - 3.6	1.3 - 3.9	0.7	0.5 - 2.3	0.8 - 2.5	0.9 - 2.4	0.8 - 5.5	0.9 - 3.5	1.2 - 2.1	2.1 - 2.9
Total P (mg l^{-1})	0.2 - 0.7	0.2 - 0.6	0.1	0.07 - 0.1	0.1 - 0.2	0.1 - 0.4	0.2 - 1.3	0.2 - 0.4	0.1 - 0.6	0.2 - 0.3
Chla (µg l^{-1})	3.5 - 40.1	15.3 - 37.2	6.8	6.8 - 14.4	15.0 - 19.6	6.5 - 12.6	3.1 - 52.9	21.5 - 52.9	9.4 - 78.6	8.0 - 62.5
Fish presence	no	no	no	x	x	x	no	x	x	x
Emergent vegetation presence	x	x	x	x	x	x	x	x	x	x
Submerged vegetation presence	x	x	x	x	x	no	x	no	no	no
2011										
Mean depth (cm)	7.7 - 44.4	8.2 - 49.8	14.2 - 58.0	7.2 - 86.2	2.8 - 50.6	15.0 - 51.8	35.2 - 45.4	32.8 - 109.8	15.2 - 96.4	33.0 - 79.4.0
Temperature (ºC)	12.5 - 25.2	13.3 - 21.1	16.5 - 27.7	12.8 - 21.5	12.5 - 21.2	12.5 - 29.1	15.1 - 26.6	15.5 - 25.7	15.5 - 25.7	12.6 - 25.8
pH	8.5 - 8.9	8.6 - 9.0	8.2 - 9.1	7.1 - 8.2	8.0 - 8.5	8.4 - 9.6	7.4 - 8.5	7.5 - 8.3	7.5 - 8.3	7.8 - 8.5
Salinity (psu)	0.4 - 8.4	0.9 - 13.8	0.5 - 4.6	0.1 - 8.7	0.3 - 4.8	0.7 - 5.3	0.3 - 0.8	0.4 - 2.3	0.4 - 2.3	0.3 - 1.9

Turbidity (NTU)	3.2 - 95.0	34.9 - 121.0	18.5 - 193.0	65 - 214	53.0 - 442.0	15.3 - 366.0	2.9 - 26.3	26.2 - 90.0	10.1 - 24.6	45.5 - 78.0
Total N (mg l^{-1})	1.1 - 5.1	1.1 - 4.4	1.0 - 5.8	1.1 - 5.0	1.9 - 8.3	0.5 - 5.3	0.7 - 8.5	0.7 - 2.6	1.0 - 2.6	0.8 - 1.6
Total P (mg l^{-1})	0.1 - 0.3	0.1 - 1.0	0.08 - 0.4	0.2 - 0.9	0.1 - 0.6	0.1 - 0.6	0.1 - 1.4	0.1 - 0.3	0.07 - 0.1	0.2 - 0.2
Chla (µg l^{-1})	2.1 - 47.9	0.1 - 121.8	1.1 - 5.1	11.8 - 44.5	12.7 - 50.4	3.3 - 76.7	2.5 - 128.4	14.5 - 64.0	5.5 - 51.5	22.5 - 92.5
Fish presence	x	x	x	x	x	x	no	x	x	x
Emergent vegetation presence	x	x	x	x	x	x	x	x	x	x
Submerged vegetation presence	x	x	x	x	x	x	x	x	x	x

Table 2. Sampling visits in new ponds and reference sites during the two studied years.

	2010	2011
New Ponds		
0N1GP	Feb-Mar-May-June	Feb-Mar-May-June
0N2GP	Feb-Mar-May-June	Feb-Mar-May-June
0N3GS	Feb-Mar-May	Feb-Mar-May
0N4GS	Feb-Mar-May	Feb-Mar-May
1N2MS	Feb-Mar-May	Feb-Mar-May
2N1PS	Feb-Mar-May	Feb-Mar-May
3N3MP	Feb-Mar-May-June	Feb-Mar-May-June
4N4PS	Feb-Mar-May	Feb-Mar-May
6N2MP	Feb-Mar-May-June	Feb-Mar-May-June
8N1MS	Feb-Mar-May	Feb-Mar-May
8N4PP	Feb-Mar-May-June	Feb-Mar-May
9N3PP	Feb-Mar-May-June	Feb-Mar-May-June
0S1GP	May	Feb-Mar-May-June
0S2GP	May	Feb-Mar-May-June
0S3GS	May	Feb-Mar-May
0S4GS	May	Feb-Mar-May-June
3S3MP	May	Feb-Mar-May-June
4S1PS	May	Feb-Mar-May
4S2MS	May	Feb-Mar-May
5S1PP	May	Feb-Mar-May-June
7S1MP	May	Feb-Mar-May
8S2PP	May	Feb-Mar-May
10S2PS	May	Feb-Mar-May-June
10S4MP	May	Feb-Mar-May-June
AO1	May	Feb-Mar-May
AO2	May	Feb-Mar-May
AC3	May	Mar-May-June
AC4	May	Mar-May-June
AE5	Mar-May-June	Feb-Mar-May
AE6	Mar-May-June	Feb-Mar-May
AE7	May	Feb-Mar-May
AE8	May	Feb-Mar-May
Reference Sites		
C.CAR	Feb-Mar-May-June	Feb-Mar-May-June
CAÑO_1	Mar-May-June	Feb-Mar-May-June
ENT_1	Mar-May-June	Feb-Mar-May-June
ENT_2	Mar-May	Feb-Mar-May-June
FAO	Mar-May-June	Feb-Mar-May-June
GUADI_1	Mar-May-June	Feb-Mar-May-June
GUADI_2	Mar-May-June	Feb-Mar-May-June
LOBO	Mar-May-June	Feb-Mar-May-June
MARILOPEZ	May	Feb-Mar-May-June
ROSALIMAN	Mar-May-June	Feb-Mar-May-June

Table 3. Results from SIMPER analyses showing the main environmental variables that contributed (%) to the monthly dissimilarity (according to ADONIS analyses) between new ponds and reference sites. Cut-off of cumulative percentage of dissimilarity set at ~50%. C= Contribution to dissimilarity. Analyses were not possible for February 2010 due to the limited number of reference ponds sampled.

	2010				**2011**			
	Feb	**Mar**	**May**	**Jun**	**Feb**	**Mar**	**May**	**Jun**
Overall dissimilarity (%)	/	9.4	9.4	11	10.0	9.7	9.8	11.8
pH		47	19	17	42	26	27	22
Turbidity						42		54
Chlorophyll-a				51	54		56	44
Emergent vegetation		30		29	27			
Submergent vegetation			47					33
Fish			35	41		57	43	

Table 4. Vegetation (emergent and submergent) and fish identify during the two studied inundations.

	New Ponds	**Reference Sites**
Emergent Vegetation		
Scirpus maritimus	x	x
Juncus subulatus	x	x
Phragmites spp.		x
Typha spp.		x
Submerged Vegetation		
Ranunculus spp.	x	x
Ruppia spp.	x	x
Chara spp.	x	x
Zanichellia spp.	x	x
Damasonium spp.	x	x
Callitriche spp.	x	x
Riella spp.	x	x
Azolla filiculoides		x
Lemna spp.		x
Fish		x
Atherina boyeri		x
Fundulus heteroclitus		x
Gambusia holbrooki	x	x
Cyprinus carpio	x	x

Table 5. Result from SIMPER analyses showing abundance of the main taxa that contributed (%) to the dissimilarity (according to ADONIS analyses) between inundations. Cut-off of cumulative percentage of dissimilarity set at ~50%. C= Contribution to dissimilarity. Significant differences in abundance (in bold) between wetland types were identified via Wilcoxon tests.

	New Ponds			**Reference Sites**		
Total macroinvertebrates						
	C	**H1 (2010)**	**H2 (2011)**	**C**	**H1 (2010)**	**H2 (2011)**
Berosus guttalis	6.21	264.7 ± 61.4	173.6 ± 41.6			
Corixidae spp. (nymphs)	5.20	317.8 ± 92.9	101.43 ± 22.6			
Chironomidae spp.	4.69	328.2 ± 76.9	375.5 ± 56.8			
Berosus affinis	4.21	50.9 ± 22.6	**136.0 ± 37.8**			
Sigara lateralis	2.80	61.7 ±1 8.3	61.1 ± 14.8			
Ochthebius viridis fallaciosus	2.51	23.4 ± 11.4	40.2 ± 14.9			
Corixa affinis	2.49	6.8 ± 2.5	23.4 ± 5.6			
Coleoptera						
Berosus guttalis	14.1	264.7 ± 61.4	173.6 ± 41.6			
Berosus affinis	10.0	50.9 ± 22.6	**136.0 ±3 7.8**			
Berosus spp. (larvae)	8.0	10.5 ± 5.1	4.8 ± 3.4			
Hemiptera						
Corixidae spp. (nymphs)	38.6	317.8 ± 92.9	101.43 ± 22.6	15.6	178.1 ± 59.4	159.4 ± 136.0
Sigara lateralis				9.6	**47.9 ± 17.2**	9.0 ± 6.1
Micronecta scholtzi				8.2	10.2 ± 7.2	16.2 ± 10.8

Table 6. Results from SIMPER analyses showing the abundance of main taxa that contributed to the monthly dissimilarity in the total macroinvertebrate community according to ADONIS analyses between new ponds and reference sites. Cut-off of cumulative percentage of dissimilarity set at ~50%. C= Contribution to dissimilarity; P = New Ponds; R = Reference Sites. Significant differences between wetland types in abundance (in bold) were identified via Mann-Whitney U tests. Analyses were not possible for February 2010 due to the limited number of reference ponds sampled.

	2010										
	Feb			**Mar**			**May**			**Jun**	
	C	**P**	**R**	**C**	**P**	**R**	**C**	**P**	**R**	**C**	**R**
Overall dissimilarity (%)				62			59				
Chirocephalus diaphanus				3.39	**8.8 ± 1.6**	2.3 ± 0.9					
Streptocephalus torvicornis				6.26	**20.1 ± 4.1**	2.3 ± 1.5					
Triops mauritanicus				3.46	2.1 ± 0.7	3.0 ± 0.8					
Oligochaeta							4.06	0.1 ± 0.1	**125 ± 57**		
Physa spp.							2.07	0	**29.6 ± 16.5**		
Aeshnidae spp.				4.30	0.1 ± 0.1	**6.7 ± 2.9**					
Berosus affinis							2.85	50.9 ± 22.6	65.8 ± 47.2		
Berosus guttalis				3.07	13.39 ± 6.9	1.0 ± 0.6	7.51	**247.6 ± 61**	56.0 ± 35		
Corixidae spp. (nymphs)							5.32	317.92 ± 59.4	178.1 ± 59.4		
Sigara lateralis							2.87	61.7 ± 18.3	47.9 ± 17.2		
Chironomidae spp.				6.89	**166 ± 32**	84 ± 7	6.11	328.1 ± 77	870 ± 470		
Tabanidae spp.				3.05	0.29 ± 0.2	**2.7 ± 0.9**					

	2011											
	Feb			**Mar**			**May**			**Jun**		
	C	**P**	**R**	**C**	**P**	**R**	**C**	**P**	**R**	**C**	**P**	**R**
Overall dissimilarity	55			53			67			60		
Chirocephalus diaphanus	2.16	6.5 ± 1.9	3.9 ± 2.5									
Streptocephalus torvicornis	7.88	**179.9 ± 45.3**	7.7 ± 4.3	4.79	**81.9 ± 25.1**	0						
Oligochaeta	1.84	0.1 ± 0.1	**10 ± 5.2**									
Physa spp.				2.23	0	**37.9 ± 22.5**	4.00	0	**233.1 ± 131.5**			
Gyraulus spp.							2.43	0	**82.1 ± 50**			

Aeshnidae spp.	2.04	0.63 ± 0.63	**6.7 ± 4.4**	2.09	0.1 ± 0.1	**6.0 ± 1.4**						
Coenagrionidae spp.							2.56	14.0 ± 3.9	58.7 ± 35.7	3.38	12.3 ± 6.3	13.5 ± 7.9
Berosus affinis							3.55	**136.0 ± 37.8**	16.7 ± 16.7	3.78	**83.3 ± 29.3**	48.9 ± 43.0
Berosus guttalis	2.33	29.8 ± 6.8	25.8 ± 7.3	6.19	184.9 ± 33	68.1 ± 36.4	5.51	**173.4 ± 41.6**	13.7 ± 9.8			
Berosus spp (larvae)							2.41	4.9 ± 3.4	**44.4 ± 33.9**			
Ochthebius viridis fallaciosus										4.97	**103.7 ± 26.6**	9.6 ± 4.0
Helophorus spp. (adults and larvae)	1.70	0.5 ± 0.2	1.7 ± 0.9									
Corixidae spp. (nymphs)	4.38	63.1 ± 16.9	52.5 ± 23.2	4.56	**147.4 ± 32**	49.1 ± 21.4	3.73	101.4 ± 22.6	159.4 ± 136.0	4.12	**194.5 ± 75.2**	7.3 ± 6.6
Sigara lateralis				2.17	36.3 ± 19.5	4.8 ± 2.6	3.32	**61.1 ±1 4.8**	9.0 ± 6.1	5.12	135.6 ± 41.3	24.0 ± 13.0
Trichocorixa verticalis										3.12	50.7 ± 14.2	10.4 ± 4.6
Chironomidae spp.	4.85	325.1 ± 33.1	232.7 ± 71.7	4.29	581.7 ± 62	615 ± 122.2	4.63	375.5 ± 56.7	522.3 ± 230.3	5.83	676.5 ± 179.0	540.2 ± 203.1

Table 7. Results from SIMPER analyses showing the abundance of main Coleoptera taxa that contributed (%) to the monthly dissimilarity (according to ADONIS analyses) between new ponds and reference sites. (/) Indicate that no significant differences emerged according to adonis analyses. Cut-off of cumulative percentage of dissimilarity set at ~50%. C= Contribution to dissimilarity; P = New Ponds; R = Reference Sites. Significant differences between wetland types in abundance (in bold) identified via a Mann-Whitney U test. Analyses were not possible for February 2010 due to the limited number of reference ponds sampled.

	2010											
	Feb			**Mar**			**May**			**Jun**		
	C	**P**	**R**	**C**	**P**	**R**	**C**	**P**	**R**	**C**	**P**	**R**
Overall dissimilarity (%)	/			85			73			84		
Berosus affinis							9.46	50.9 ± 22.6	65.8 ± 47.2	15.10	127.6 ± 32.6	30.8 ± 21.8
Berosus guttalis				16.87	13.4 ± 6.9	10 ± 0.6	19.54	**264.7 ± 61.4**	56.1 ± 34.9	15.61	**141.1 ± 75.5**	1.3 ± 1.3
Berosus spp. (larvae)							17.11	10.5 ± 5.1	5.0 ± 3.2	22.84	0	30.7 ± 28.7
Bagous spp.				11.59	0.1 ± 0.1	**3.7 ± 2.9**						
Agabus spp. (larvae)				9.28	**1.3 ± 0.6**	0						
Haliplus andalusicus				9.63	0.6 ± 0.4	1.8 ± 0.9						

	2011											
	Feb			**Mar**			**May**			**Jun**		
	C	**P**	**R**	**C**	**P**	**R**	**C**	**P**	**R**	**C**	**P**	**R**
Overall dissimilarity (%)	52			/			66			38		
Berosus affinis							9.04	**136.0 ± 37.8**	16.7 ± 16.7	13.58	**83.3 ± 23.8**	48.9 ± 43.0
Berosus guttalis	6.55	29.8 ± 6.8	25.8 ± 7.3				13.56	**173.6 ± 41.6**	13.7 ± 9.8			
Berosus hyspanicus	2.95	0	**1.7 ± 1.0**									
Berosus spp. (larvae)							13.34	4.9 ± 3.4	**44.4 ± 33.5**			
Agabus spp. (larvae)	5.62	2.1 ± 0.7	3.7 ± 2.7									
Ochthebius viridis fallaciosus										13.58	**103.7 ± 30.9**	9.6 ± 4.0
Haliplus andalusicus	5.28	1.8 ± 0.6	5.0 ± 3.7									
Helophorus spp. (adults and larvae)	4.95	0.5 ± 0.2	1.7 ± 0.9									
Hydroghlyphus gemini										10.29	11.6 ± 9.7	11.0 ± 5.7
Laccophilus spp. (larvae)	4.01	1.7 ± 0.5	1.2 ± 0.9				6.04	5.7 ± 2.8	44.4 ± 34.7			

Table 8. Results from SIMPER analyses showing the abundance of main Hemiptera taxa that contributed (%) to the monthly dissimilarity (according to ADONIS analyses) between new ponds and reference sites. Cut-off of cumulative percentage of dissimilarity set at ~50%. C= Contribution to dissimilarity; P = New Ponds; R = Reference Sites. Significant differences between wetland types in abundance (in bold) were identified via Mann-Whitney U tests.

	2011											
	Feb			**Mar**			**May**			**Jun**		
	C	**P**	**R**	**C**	**P**	**R**	**C**	**P**	**R**	**C**	**P**	**R**
Overall dissimilarity (%)	38			47			71			65		
Corixidae spp. (nymphs)	9.54	63.1 ± 16.9	52.5 ± 23.2	8.33	**147.4 ± 32**	49.2 ± 21.4	11.55	101.4 ± 22.6	159.4 ± 136.0	10.05	**194.5 ± 94.2**	7.3 ± 6.6
Corixa affinis	5.38	0.1 ± 0.1	0.2 ± 0.2		9.1 ± 3.0	3.5 ± 1.8	9.27	23.4 ± 5.6	14.6 ± 8.2			
Micronecta scholtzi				6.14	0	**11.2 ± 7.2**				7.45	0.4 ± 0.3	17.8 ± 15
Sigara lateralis				8.12	36.3 ± 19.6	4.8 ± 2.6	11.02	**61.1 ± 14.8**	9.0 ± 6.1	9.35	135.7 ± 50.3	24.0 ± 13.0
Trichocorixa verticalis	6.19	0.3 ± 0.1	**2.7 ± 1.2**							8.88	50.7 ± 17.1	10.4 ± 4.6
Notonectidae spp. (nymphs)							6.19	**14.0 ± 3.3**	1.9 ± 1.4			

Synthesis

In the 18 years since its first detection in the Iberian Peninsula *T. verticalis* has increased its area of distribution not only within Doñana, but also in other areas of great conservation interest such as Ramsar wetlands and nature reserves in Andalucia. The present thesis provides valuable insight into the factors that contribute to make this species so successful in its introduced range. We used both experimental and observational approaches to consider the role of four main determinants of successful invasions: plasticity, resource competition, parasites and facilitative interactions among invaders. Aimed at the general understanding of this invasion, we also investigated the macroinvertebrate communities of newly created ponds that housed breeding populations of this invasive species to shed light on the potential consequences on native communities.

Previous literature illustrated the ability of *T. verticalis* to cope with high salinity in both its native and invaded range. This trait is assumed either to facilitate its colonization and the dominance in the sites it invaded, or to confer competitive advantage over native corixids, especially in the face of global change. Differences in performance and plasticity between native and invasive species have often been considered of particular importance during the invasion process. Based on this, but also taking into account the potential synergistic or additive effects of temperature and salinity on controlling insect physiological tolerance, in ***Chapter 1*** we examined whether the ecophysiology of this alien species favours its spread in the Iberian Peninsula and its dominance in saline areas. We experimentally compared several indicators of upper and lower thermal sensitivity of individuals of *T. verticalis* and the native *Sigara lateralis* acclimated to different conditions of temperatures and salinities. We showed that the physiological responses to heating, cooling and freezing differed between native and invasive corixids. Although being less thermally tolerant than native species, *T. verticalis* showed broader physiological plasticity when exposed to different conditions of temperatures and salinities. In addition, *T. verticalis* increased its tolerance to both heat and freezing following exposure to high conductivities. Furthermore, the osmoregulatory ability of *T. verticalis* makes this species able to spend the cold season in saline wetlands in southern Spain, where it can achieve a continuous reproduction, and this factor could explain its dominance in permanent saline ponds. Translated to a climate change perspective the greater plasticity of *T. verticalis* makes it better than native species to survive temperature and salinity fluctuations, a factor that may further facilitate its spread in the future.

Resource competition has long been regarded as one of the major mechanisms responsible for successful invasions, and invasive species are frequently considered superior competitors. If competition between native and invasive species occurs, the outcome of their interactions (species exclusion or coexistence) depends on the degree of resource/niche overlap, which can be context specific. The high voracity of *T. verticalis* in the native range and the morphological similarity of mouthparts within Hemiptera suggest that it may have a high impact on native corixids through resource competition. In ***Chapter 2*** we used stable isotopes of carbon (C) and nitrogen (N) applied to experimental and field data, to explore niche partitioning between *T. verticalis* and native co-occurring corixids in permanent (*S. lateralis)* and temporary ponds (*S. lateralis* and *Sigara scripta*) in Doñana. We also explored the trophic ecology of *T. verticalis* in permanent saline waters, where it has stable reproductive populations. As expected, there was a clear change in trophic interactions between native and invasive corixids in both waterbody types. In permanent ponds, where we assumed that consumers and resources were all in equilibrium, the native and invasive corixids had different isotopic values and no trophic overlap. This suggests that strong resource partitioning facilitates their coexistence. In particular, the consistently lower $\delta^{15}N$ of the invasive species suggests that it may be feeding at a lower trophic position and relaying more on herbivory (i.e., periphyton) than its native competitors. However, in temporary sites, with high corixid densities and characterized by high disturbance and superabundant food of low diversity, the native and invasive corixids showed some degree of niche overlap, and a slight reduction in trophic level for the native species. This suggests that the high dietary plasticity of the native species is the mechanism behind their coexistence in temporary ponds. Overall, these findings indicate that competition among corixids is not equal between habitats that differ in stability and types and abundances of local food sources.

It is well known that hemipterans host a diverse community of parasites, including water mites (Hydracarina) that inhabit almost all fresh and brackish aquatic environments. *T. verticalis* may either be less parasitized than native species because it loses its natural parasites during the introduction process, or it can be more parasitized because it represents a more suitable host for native parasites. In addition, new parasites may also have been introduced with *T. verticalis,* with potential negative effects on native species. In ***Chapter 3***, we explored the potential role of water mites in the invasion dynamics of *T. verticalis.* We compared infestation levels of larval water mites between native (*S. lateralis* and *S. scripta*) and exotic corixids along a salinity gradient, taking also into account the effect of the host's size for parasite acquisition. Samples from a specific sampling were used to obtain the calculation of mite infection rates as accurately as possible. In saline wetlands where *T.*

verticalis dominates, water mites were absent, in contrast to temporary waters where two mite species were found to affect corixids (*Eylais infundibulifera* and *Hydrachna skorikowi)*. For both of them there was a negative association between prevalence and salinity. Overall, the total parasite prevalence, total parasite abundance and mean intensity of each water mite were higher in *T. verticalis* than in native corixids. The greater prevalence of *H. skorikowi* in T. verticalis respect to *S. lateralis* and of *E. infundibulifera* respect to both native corixids points toward a case of parasite acquisition and a reencounter between host and parasites in the invaded area, respectively. Water mites may have different effects on corixids, including reducing flight ability and reproductive success or competing them for food. Therefore, parasites may have a role in explaining why in low salinity waters, where T. verticalis suffers higher susceptibility to infection compared to native corixids, the species is not particularly successful. On the other hand, in saline waters the absence of water mites may partly explain the abundance and dominance of T. verticalis.

In a multiple invaded scenario, the presence of one invader may potentially promote the establishment and spread of new invaders, especially if it shares a co-evolutionary history with them. It has been shown that facilitative interactions are as frequent as any other biotic interaction. As a consequence, the co-occurrence of exotic predators and exotic prey can, for example, heavily impact native prey by hyperpredation. Based on this premise and assuming that only organisms with specific and effective anti-predatory mechanisms are likely to coexist with exotic predators, in ***Chapter 4*** we investigated whether the spread of the alien water boatman *T. verticalis* in the Iberian Peninsula is related to reduced mortality from predation compared with native Corixidae, especially since *T. verticalis* shares a common native range in North America with the invasive fishes *Gambusia holbrooki* and *Fundulus heteroclitus.* By using an experimental approach we compared the proportion of corixids (either invasive or native) eaten by the two exotic fish and by native Odonata larvae. We hypothesized that predator responses to different prey depend on shared co-evolutionary history, and that differences in predation also depend on predator and prey size. Our results provide no evidence that the invasion of *T. verticalis* has been promoted by lower predation rates compared to native corixids. We were able to show that both alien and native corixids suffered similar, high predation rates by *Fundulus* and Odonata larvae. Instead, when both corixids were mixed together, *T. verticalis* were eaten more by Odonata larvae, as the result of their smaller size. We suggest that *Fundulus* and the native Odonata, which are more common in permanent saline waters and low salinity ponds respectively, might have a heavy impact on the invasive corixids. However, it seems possible that in saline waters the presence of alternative prey for *Fundulus*, and the presence of top predators that feed on this exotic fish can reduce the extent of predation on *T. verticalis.* Instead, in low salinity ponds *T. verticalis* may suffer particularly high predation rates from native Odonata larvae, which may provide biotic resistance to their invasion and may partly explain why this species is particularly successful in saline habitats where Odonata are rare.

As in many other countries, wetland ecosystems in the Iberian Peninsula are subject not only to habitat loss and degradation, but also to multiple biological invasions. In recent years, restoration projects have become common practice aimed at re-establishing ecosystem functions and reversing biodiversity losses worldwide. In some cases invasive species motivated restoration projects, but sometimes they were also the causes of their failure. In ***Chapter* 5**, we investigated the value of the Caracoles restoration project in supporting macroinvertebrates during two consecutive inundations. *T. verticalis* is known to have reproductive populations in these new ponds. We compared the temporal dynamics of two dominant groups of active dispersers (Coleoptera and Hemiptera) with the whole aquatic macroinvertebrate community (a mix of active and passive dispersers) to shed light on dispersal constraints during ecosystem recovery. Macroinvertebrate richness and diversity of new ponds matched those in reference sites and abundance was even higher, but they showed different community composition. As expected, dispersal limitation affected the recovery of the whole community, but contrary to our expectations new ponds contributed equally to the overall nestedness than reference sites. We suggest that new ponds provide complementary habitats suitable for species with or without specific preference for them. Although most of the differences between waterbodies were the result of their environmental differences, these differences may also be partly due to the different distribution of invasive species between waterbodies and hydroperiods (including alien fish as well as *T. verticalis*).

Integrating results

The results of this thesis shed light on the role of salinity for the invasion success of *T. verticalis.* The dominance of this invader in saline waters, but its absence from fresh waters where native corixids are dominant, seems the result of the combination between its ability to maintain continuous reproduction and development in stressful situations (***Chapter 1***), for example, during the cold season (Jack-of-all-trades scenario *sensu* Richards et al., 2006); the reduced probability to encounter less halotolerant natural enemies such as parasites and predatory Odonata larvae (***Chapter* 3** & **4**); and the higher probability to find high quality periphyton (***Chapter* 2**). In addition, in stable permanent saline ponds fish may act as keystone predators, preventing the dominant competitor from monopolizing the major resources (Brucet et al., 2005), thus the overall diversity of food should be greater than in temporary sites. In such conditions, native and invasive corixids can co-exist through resource partitioning (***Chapter* 2**), and predators that include corixids in their diet may encounter alternative prey or may be low in abundance owing to limitation by their own predators (***Chapter* 4**). This suggests that saline waters act as sources of *T. verticalis* for the surrounding freshwater habitats in Doñana and elsewhere.

We found good evidence that, to date, *T. verticalis* does not represent a serious threat for co-occurring native species in temporary sites. As emerged from ***Chapter* 5** the presence of *T. verticalis* within the newly created ponds in Caracoles did not affect the successful recovery of macroinvertebrates, including corixids (***Chapter* 5**). However, in these waters this could be the result of the greater rate of parasitism infection that *T. verticalis* suffered respect to native Corixids (***Chapter* 3**) and the greater predation pressure exerted by Odonata larvae on them (***Chapter* 4**). Both these factors may have prevented *T. verticalis* to exert a strong impact on native species by increasing their mortality rates, limiting their population growth and changing the outcome of species relationships. In addition, the stressful conditions of high temperature and low salinity that are likely to favour the invader over native corixids are generally concentrated in a short phase within the inundation, when the abundance of food is high even if its diversity is low (***Chapter* 2**).

However, our results also suggest a risk of more complex interactions with native corixids in the future. In ***Chapter* 5**, we suggested that differences in the abundance and in the number of sites colonized by *T. verticalis* between years might have had strong effects on the patterns of macroinvertebrate recovery that we have detected in the Caracoles estate, especially for corixids. *T. verticalis* colonized a higher number of sites during the second studied year. Probably the environmental differences (e.g., water depth, salinity) between the two inundations are responsible for such differences. Climate change will affect aquatic systems by warming waters, increasing evapotranspiration rates and salinity (Moss et al., 2009). As suggested in ***Chapter* 1**, these changes are expected to favour *T. verticalis* spread since it will be better able than native species to cope with higher salinities and climate fluctuations. At the same time, increases in water salinity may filter out the most sensitive taxa including parasites and Odonata larvae. Alternatively, as in the case of epibiotic mites infecting the invasive crab *Eriocheir sinensis* (Normant et al., 2013), the further expansion predicted for *T. verticalis* (Guareschi et al., 2013) may cause a disease impact on native corixid populations via the spillback of native parasites (Daszak, Cunningham, & Hyatt, 2000; Tompkins & Poulin, 2006). Climate change can also decrease aquatic system productivity (O'Reilly, Alin, Plisnier, Cohen, & McKee, 2003), which implies that competition for food between native and invasive species may become exacerbated (***Chapter* 2**). L'Mohdi and co-workers (2010) suggested that the presence of *T. verticalis* affected the halophilic *Sigara selecta*. In Doñana *S. selecta* already seems to be much rarer than before, and in our study sites *S. selecta* was absent, so we cannot rule out the possibility that this absence was induced by a strong competition with the invasive corixid. The future expansion of *T. verticalis* predicted by bio-climatic models (Guareschi et al., 2013) suggests that the presence of *S. selecta* in SW Spain may be seriously threatened by this invasion. Similarly, *T. verticalis* might also affect the other native species in the future, when salinities and temperatures increase.

Future perspectives

Overall, this thesis helped to shed light on the mechanisms and processes explaining the success of *T. verticalis* as an invasive species in saline waters, and the possible consequences of this invasion for native fauna in less saline sites. However, several new questions that emerged during this study need to be investigated in the future. Here I suggest several research lines that may help to further our understanding of this invasion process.

It will be interesting, in my opinion, to introduce a comparative biogeographical approach to this invasion, applying molecular analyses and an invasion genetics approach. This should allow identification the subspecies of *T. verticalis* arrived in the Iberian Peninsula, from where it has spread. The physiological performance of *T. verticalis* should also be compared between the native and invaded range, so as to understand the role of adaptive evolutionary changes in this invasion. It would also be interesting to examine whether the distinct populations outside its native range (i.e. in the Iberian Peninsula, Morocco, South Africa, etc) have a common origin.

A better understanding of corixid diet seems crucial, especially for predicting the potential impact of *T. verticalis* on the foodweb. I suggest the use of amino acid isotopes analyses instead of bulk tissue isotopes analysis. Despite being much costly, this technique can bypass some of the problems we encountered using the bulk tissue approach. Since our results suggested that *T. verticalis* might compete for food with native species under prolonged stressed conditions, I suggest the use of manipulative experiments of salinity, food (diversity and availability) and consumer density to confirm this hypothesis, using also native species not included in this study, such as *Sigara selecta*.

Since the higher levels of parasite infection in *T. verticalis* respect to native corixids, and its high dispersal ability, make it plausible that *T. verticalis* amplifies the infections on native corixids, experimental tests may help to elucidate this hypothesis. In addition, as mentioned above, parasites could affect hosts in different way including reducing fecundity and affecting individual growth. It would be interesting to compare the effects of parasite infections between native corixids and *T.verticalis,* and to study whether parasite infections alter their behaviour or morphology, to elucidate the role of parasites during invasion processes.

Regarding the effect of *T. verticalis* on the invaded community, our results showed different patterns of recovery in Hemiptera composition between years. It would be helpful to conduct a mesocosm study that experimentally tests the impact of *T.verticalis* during community assembly, particularly if the invasion sequence affected the community structure.

Finally, there is the need, in my opinion, for long-term monitoring of macroinvertebrates in areas thought to be suitable for *T. verticalis* invasion but which have not yet been invaded or in which this species is still rare, so as to have more accurate information on the changes in the invaded community that this species can produce after its arrival through time. This would include any saline wetland east of Gibraltar, since the species has not yet been detected further east than Barbate.

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General Conclusions

Chapter 1

The physiological responses to heating, cooling and freezing differ between the invasive *T verticalis* and the native *S. lateralis.* Although the native species has a broader thermal range, the alien corixid has greater plasticity to heat, cold and freezing.

T. verticalis performs particularly well at higher salinities and temperatures. In particular, its tolerance to both heat and freezing increases following exposure to high conductivities.

The osmoregulatory ability in *T. verticalis* might enhance its resistance to cold stress, which in turn may allow the invasive species to spend the cold season in saline wetlands where it achieves continuous reproduction and development.

Chapter 2

Trophic interactions between native and invasive corixids change between habitats that differ in water stability and in the types and abundances of local food sources.

Resource partitioning between native and invasive corixids is strong in stable waters, where *T. verticalis* may be feeding at a lower trophic position and relying more on herbivory than its native competitors. In contrast, niche overlap between native and invasive corixids exists in temporary waters, and the native species seems to occupy a slightly lower position compared to stable waters. In combination, these results indicate that competitive interactions can exist between them.

In stable permanent ponds the invasive species has a periphyton-based diet and can be assigned to the grazer guild, especially the nymphs.

Chapter 3

In saline wetlands, where *T. verticalis* dominates, water mites are absent. In contrast, in temporary waters *T. verticalis* is infected by both species of water mites found in this study (*Hydrachna skorikowi* and *Eylais infundibulifera).*

Total parasite prevalence, total parasite abundance and mean infection intensity of water mites are higher in *T. verticalis* than in native corixids.

The greater prevalence of *H. skorikowi* in *T. verticalis* compared with *S. lateralis* points to a case of parasite acquisition. On the other hand, the greater prevalence of *E. infundibulifera* in *T. verticalis* respect to both *S. lateralis* and *S. scripta* does not exclude the possibility that this parasite came with *T. verticalis*, although it is more likely that they have reencountered in the invaded area.

The higher susceptibility to parasites compared with native species probably limits *T. verticalis* invasion in natural wetlands of low salinity.

Chapter 4

T. verticalis does not suffer lower predation rates compared to native corixids. Predation rates do not depend on whether corixids encounter predators sharing a common native range or predators of a different origin.

Odonata larvae distinguish between alien and native corixids on the basis of their body size. The smaller *T. verticalis* suffers higher predation pressure than the larger *S. lateralis*. This may partly explain why this species is particularly successful in saline habitats where Odonata are rare.

Chapter 5

New ponds created during a wetland restoration project match or even surpass the levels of local invertebrate richness, diversity and abundance 6-7 years after restoration, despite having different and more homogeneous abiotic conditions than reference sites.

Passive dispersers (i.e., Gastropoda and Oligochaeta) have so far failed to colonize new ponds, suggesting that dispersal limitation is more important than environmental constraints for the recovery of these ponds over a short time frame.

New ponds house slightly less diverse communities than reference sites, but new ponds and reference sites contribute equally to the global nestedness. New ponds contain taxa not found in reference sites, indicating their complementary functions as well as their value as habitats for biodiversity conservation.

Differences in the abundance and distribution of T. *verticalis* between new and reference ponds among years might explain the annual differences found for Hemiptera species composition, especially towards the end of the hydroperiod, when environmental filters become stronger (e.g. temperature and salinity increase).

In summary, our results indicate that *T. verticalis* is a successful invader in saline waters, but it is rare in fresh waters, because in saline waters its physiological performance improves, it is released from natural enemies (native parasites and predators) and it does not compete for food with native corixids.

Conclusiones Generales

Capítulo 1

La respuesta fisiológica de la especie invasora *T. verticalis* al calor, al frío y al congelamiento es diferente a la respuesta de la especie autóctona *S. Lateralis*. A pesar de que la especie nativa tiene una mayor tolerancia térmica, la especie exótica tiene una mayor plasticidad fisiológica al calor, al frio y al congelamiento.

La respuesta fisiológica de *T. verticalis* es mas efectiva a condiciones de elevada salinidad y temperatura. En concreto, su tolerancia térmica al calor y al frío aumenta tras ser aclimatada a una elevada salinidad.

La capacidad de regulación osmótica de *T. verticalis* podría también incrementar su resistencia al frio, y a la vez facilitar su permanencia durante el invierno en humedales salobres donde puede reproducirse y desarrollarse de forma contínua.

Capítulo 2

Las interacciones tróficas entre el coríxido exótico y los nativos varían en hábitats de diferente hidroperiodo y que tienen diferente diversidad y abundancia de recursos tróficos.

En humedales permanentes, los coríxidos nativos y exótico usan distintos recursos tróficos. La especie exótica parece alimentarse en un nivel trófico inferior, y depender más de recursos vegetales en comparación a sus competidores nativos. De forma contraria, en humedales temporales, los coríxidos nativos y exótico muestran un solapamiento en sus nicho isotópico, y la especie nativa se alimenta a un nivel trófico inferior que en humedales permanentes. La combinación de estos resultados sugieren que pueden existir interacciones competitivas entre ellos.

En humedales permanentes la especie invasora se alimenta principalmente de perifiton y podría considerarse un “grazer”, sobre todo las ninfas.

Capítulo 3

En humedales salobres donde la especie invasora *T. verticalis* es el coríxido dominante, no se encontraron ácaros acuáticos. Contrariamente, en los humedales temporales *T. verticalis* es infectado por las dos especies de ácaros acuáticos encontrados durante este estudio *(Hydrachna skorikowi* and *Eylais infundibulifera)* .
La prevalencia total de parásitos, la media total de sus abundancias de infección y la media de infección de cada parásito es mayor en *T.verticalis* que en los coríxidos nativos.

La elevada prevalencia de *H. skorikowi* en *T. verticalis* comparada a la de *S. lateralis* indica un caso de adquisición de parásitos. De forma contraria, la mayor prevalencia de *E. infundibulifera* en *T. verticalis* comparado a la de *S. lateralis* y *S. scripta* no excluye la posibilidad de que este parásito haya llegado junto con *T. verticalis*, aunque parece ser mas probable que se hayan reencontrado en la zona invadida.

Es probable que la mayor susceptibilidad de *T. verticalis* a ser parasitado comparado con los coríxidos nativos limiten la invasión de esta especie en humedales naturales de menor salinidad.

Capítulo 4

T. verticalis no sufre una menor tasa de depredación en comparación con los coríxidos nativos. El origen de los depredadores (nativo o exótico) no influye en la tasa de depredación de los coríxidos.

La larva de Odonato es capaz de distinguir entre coríxidos exótico y nativo debido a la diferencia de tamaño. *T. verticalis* (siendo mas pequeño) tiene una tasa de depredación más elevada que *S. Lateralis.* Esto podría explicar en parte el porqué *T. verticalis* tiene más éxito en hábitats salobres donde las larvas de Odonato son escasas.

Capítulo 5

Tras 6-7 años de su creación los lucios artificiales presentan el mismo nivel de riqueza taxonómica y diversidad, pero una mayor abundancia que los sitios de referencia, aunque sus características ambientales sean diferentes y más homogéneas en comparación con éstos últimos. Los dispersantes pasivos (i.e., Gastropoda and Oligochaeta) no han colonizado hasta el momento los lucios artificiales, lo que sugiere que las limitaciones relacionadas a la dispersión son más importantes que las que imponen las características ambientales en nuestro sistema de estudio. Los lucios recién creados tienen comunidades menos diversas que los sitios de referencia, pero contribuyen de forma similar al patrón de anidamiento general de las comunidades. Sin embargo, en los lucios recién creados se han encontrado taxa no presentes en los sitios de referencia, indicando así su función complementaria y su valor como hábitat para la conservación de la biodiversidad.

La diferencia anual en la abundancia y distribución de *T. verticalis* entre los nuevos lucios y los sitios de referencia, podrían explicar el diferente patrón anual encontrado en la composición de la especies de Hemípteros, especialmente hacia el final del periodo de inundación cuando la condiciones ambientales son más rigurosas (e.g. incremento de temperatura y salinidad).

Para concluir, los resultado de esta tesis indican que *T. verticalis* tiene mayor éxito en los cuerpos de agua más salobres debido a que su tolerancia fisiológica es más efectiva, donde está libre de parásitos, depredadores y tampoco compite con los coríxidos nativos por los recursos tróficos.

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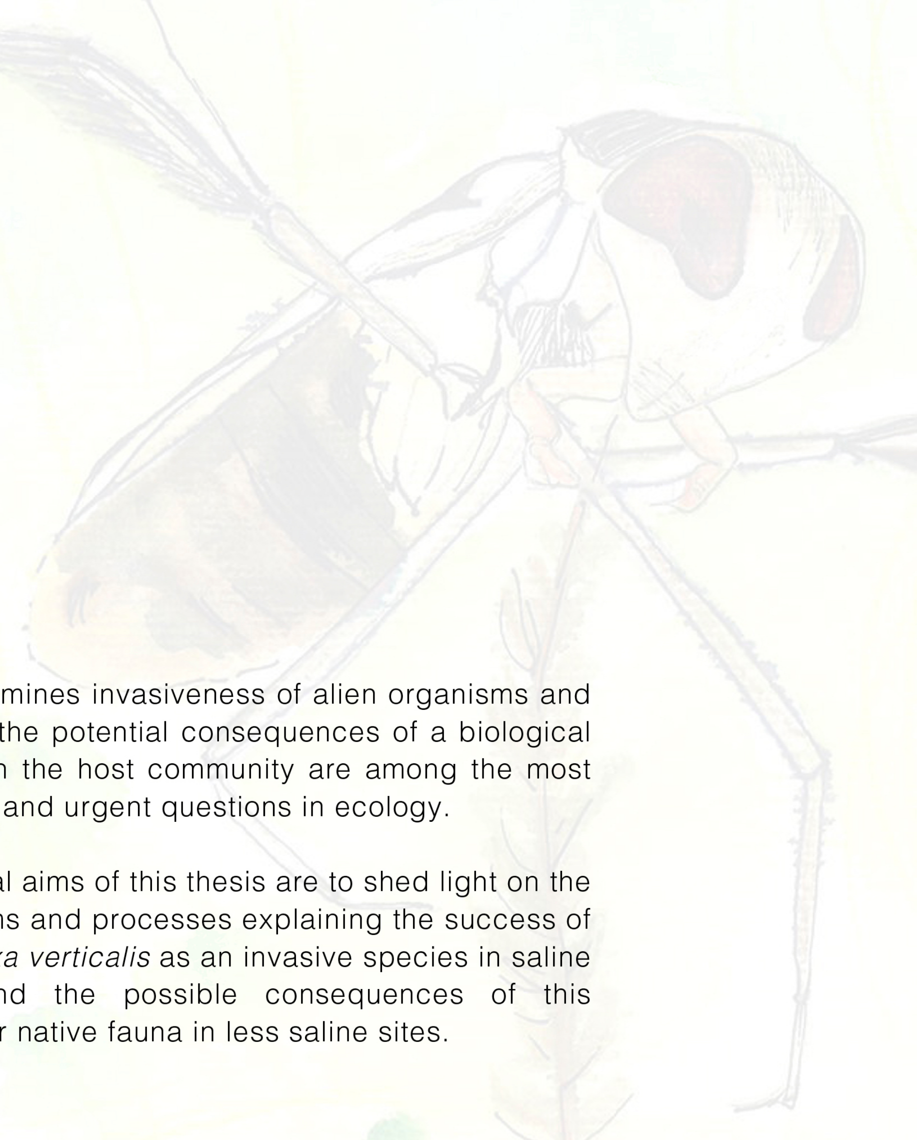

What determines invasiveness of alien organisms and which are the potential consequences of a biological invasion on the host community are among the most interesting and urgent questions in ecology.

The general aims of this thesis are to shed light on the mechanisms and processes explaining the success of *Trichocorixa verticalis* as an invasive species in saline waters, and the possible consequences of this invasion for native fauna in less saline sites.

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