Freshwater Biology

Secondary dispersal of plants and invertebrates by gulls feeding on alien crayfish

Journal:	*Freshwater Biology*
Manuscript ID	FWB-P-Sep-17-0467.R2
Manuscript Type:	Standard Paper
Date Submitted by the Author:	14-Jan-2018
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Keywords:	Invasive species < Applied Issues, Fresh waters < Habitat, Dispersal < Process / Approach / Methods, Birds < Taxonomic Group / Assemblage, Invertebrates < Taxonomic Group / Assemblage, Higher plants < Taxonomic Group / Assemblage, Lower plants < Taxonomic Group / Assemblage

Crayfish invasion facilitates dispersal of plants and invertebrates by gulls

Short title: Secondary dispersal by gulls

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Keywords: biological invasions, endozoochory, epizoochory, *Procambarus clarkii*, seeds

Summary

1. The red swamp crayfish (*Procambarus clarkii*), originally from North America, is one of the world's worst aquatic invaders. It is a favoured prey item for waterbirds, but the influence of this novel predator-prey relationship on dispersal of other organisms has not previously been considered. We investigated the potential for dispersal of plants and invertebrates by migratory waterbirds feeding on alien *P. clarkii* in European ricefields at harvest time.

2. In November-December of 2014-2015, we collected propagules from the outside of 13 crayfish captured as they moved out of ricefields during harvest in Doñana, south-west Spain. We also collected excreta (N = 76 faeces, 14 pellets) of lesser-black backed gull (*Larus fuscus*).

3. We recorded diaspores from at least 11 plant species (161 seeds from 10 angiosperm taxa, and 14 charophyte oogonia) on the outside of crayfish, together with 54 eggs from eight aquatic invertebrate taxa. Adults and juveniles of at least nine microcrustaceans, including the alien ostracods *Hemicypris reticulata* and *Ankylocythere sinuosa*, were also recovered from crayfish. No intact propagules were present in the digestive system of the crayfish.

4. Contents of regurgitated pellets confirmed *P. clarkii* as the main food item for gulls. Diaspores from at least 12 plant species (154 seeds from 11 angiosperm taxa, and 17 charophyte oogonia) were recovered from gull excreta, together with 129 eggs of 12 aquatic invertebrate taxa. A statoblast of the alien bryozoan *Plumatella vaihiriae* was found in gull faeces. Seven of the plant species are important agricultural weeds, and two are alien to Spain. Diaspores from six plant taxa were germinated, confirming viability. These propagules were from a similar set of plants and invertebrates to those found on the outside of crayfish, suggesting that propagules in gull excreta were ingested inadvertently with their crayfish prey.

5. Ricefields constitute a major artificial aquatic habitat covering an increasing proportion of the world's land surface, and typically support native or alien crayfish. Crayfish invasion can lead to novel secondary dispersal pathways for plants and invertebrates through interactions with their predators, promoting the expansion of alien and native species (including weeds) through long-distance dispersal via migratory waterbirds, and increasing connectivity of organisms between artificial and natural ecosystems. This represents a previously overlooked impact of crayfish invasion on ecosystem services.

Introduction

Many plants and invertebrates are able to disperse with vertebrate vectors, although current knowledge of these interactions remains limited (Tesson et al., 2015). Propagules (mostly seeds or resting eggs) can be dispersed internally (in the digestive system of a vector, i.e. “endozoochory”) or externally (attached to the body of a vector, i.e. "epizoochory" or "ectozoochory"). Endozoochory by frugivores is the relatively well studied, and illustrates how dispersal interactions are major determinants of the composition and gene flow in biological communities, and a vital part of the architecture of biodiversity, or “interactome” (García, Klein & Jordano, 2017). Increasingly, it is becoming clear that waterbirds are key vectors of plants lacking a fleshy fruit, as well as of a range of invertebrate groups (Coughlan, Kelly, Davenport & Jansen, 2017; Soons, Brochet, Kleyheeg & Green, 2016; Green, 2016; Valls et al., 2017). This often happens because waterbirds feed directly on seeds or invertebrates but only digest a fraction of them, and this can be considered as “primary dispersal”.

Such dispersal is vital for metacommunity dynamics, and for maintaining connectivity between populations in isolated catchments and in fragmented landscapes (Tesson et al. 2015; Green, Soons, Brochet & Kleyheeg, 2016). Migratory waterbirds are also vectors for the long-distance dispersal (LDD) that allows species to achieve widespread distributions, and to respond to global change (Nathan et al., 2008; Green et al., 2016). Darwin (1872) showed experimentally that “secondary dispersal” may also be important, in which birds predate on organisms such as fish which have previously ingested seeds or other propagules. Secondary plant dispersal by birds of prey and carnivorous mammals has been demonstrated (Nogales, Quilis, Medina, Mora & Trigo, 2002; Hämäläinen et al., 2017), yet such secondary dispersal processes by waterbirds have hardly been investigated (Green, Soons, Brochet & Kleyheeg, 2016). Recently, however, cormorants have been shown to disperse a variety of propagules previously ingested by fish (van Leeuwen, Lovas-Kiss, Ovegård & Green, 2017). Secondary dispersal can provide several benefits to dispersed taxa, since the secondary vector may have higher mobility and greater capacity for LDD. In addition, propagules that may not survive gut passage in one organism may still be dispersed if a predator ingests this organism, and the propagules within, before gut passage has been completed (Hämäläinen et al., 2017). Studies of secondary dispersal by waterbirds are therefore required to assess its importance in the maintenance of biodiversity and the spread of alien species, and its role in the interactome in aquatic ecosystems (Reynolds, Cummings, Vilá & Green, 2017; van Leeuwen, Lovas-Kiss, Ovegård & Green, 2017).

The red swamp crayfish (*Procambarus clarkii*) is increasingly widespread as an alien species, and is considered to be amongst the world's worst aquatic invaders owing to major impacts on aquatic ecosystems and ecosystem services (Geiger, Alcorlo, Baltanas, & Montes, 2005; McLaughlan, Gallardo, & Aldridge, 2014). In Europe, it was first introduced into Doñana, SW Spain from Louisiana, USA in 1973. Although the influence of this invasion on dispersal interactions has yet to be fully examined, crayfish have been shown to act as vectors of dispersal for the eggs or juveniles of aquatic invertebrates that can adhere to their external surfaces, or survive ingestion and gut passage (Moore & Faust, 1972; Pérez-Bote, Del Viejo, García & Rodríguez, 2005). In Europe, for example, the exotic ostracod *Ankylocythere sinuosa* (Rioja, 1942) and the branchiobelid *Xironogiton victoriensis* (Gelder & Hall, 1990) have been found to co-occur with the widely distributed *P. clarkii*, including those populations located across the Iberian Peninsula (Diéguez-Uribeondo, Temiño & Muzquiz, 1997; Gelder, 1999; Aguilar-Alberola et al., 2012).

Procambarus clarkii is often abundant in habitats used by large numbers of migratory waterbirds, such as European ricefields (Pernollet et al., 2015). The Doñana wetlands in Spain are one of the most important wintering sites for waterbirds in the western palearctic (Rendón, Green, Aguilera & Almaraz, 2008), and support many bird species that can predate on *P. clarkii* (including grey heron *Ardea cinerea*, white stork *Ciconia ciconia*, little egret *Egretta garzetta*, glossy ibis *Plegadis falcinellus*, and lesser-black backed gull *Larus fuscus*, Tablado, Tella, Sánchez-Zapata & Hiraldo, 2010). Doñana includes up to 37,000 ha of ricefields (Green, Bustamante, Janss, Fernández-Zamudio & Díaz-Paniagua, 2017), where bird numbers peak at the time of rice harvest, with many of the same birds switching to natural wetlands in Doñana and other parts of Andalusia during the rest of the wintering period (Rendón et al., 2008, Bouten, Baaij, Shamoun-Baranes & Camphuysen 2013). Gulls (Laridae) are opportunistic and omnivorous birds that often feed on alien crayfish (Christel, Navarro, del Castillo, Cama & Ferrer, 2012, Mortimer et al., 2012, Gyimesi et al., 2016). Equally, gulls also consume grains and other seeds, and can be important vectors for dispersal of native and alien plants (Green, 2016; Green, et al., 2016). However, there have been no previous reports of secondary dispersal by gulls. *Larus fuscus* is a migratory species with an increasing population wintering in southern Spain but breeding in northern Europe (Bouten, et al. 2013), and up to 15,000 are present in the Doñana ricefields at harvest time (Rendón et al., 2008).

Gulls can be observed feeding in muddy fields that have been drained for harvesting. When caught, crayfish are covered in mud (Fig. 1) owing to absence of standing water, and are swallowed quickly to avoid kleptoparasitism, i.e. before they can be stolen by other birds (Oro & Martínez-Vilalta, 1994).

In this study, we evaluate the potential for secondary dispersal of propagules by gulls that are feeding on crayfish in the ricefields of the Doñana area. Our aims were to investigate what kinds of seeds and invertebrates are transported by *P. clarkii* and *L. fuscus*, and determine whether organisms dispersed by gulls are ingested together with crayfish prey (i.e. carried within or upon crayfish). We compared propagules dispersed by gulls in pellets and faeces, because the latter are likely to be retained in the gut for longer, with more chance of undergoing LDD (Nogales, Medina, Quilis & González-Rodríguez, 2001). We also investigated whether taxa dispersed included alien species or agricultural weeds, whose dispersal by migratory birds may constitute an ecosystem disservice (Green et al., 2016, Farmer, Webb, Pierce & Bradley, 2017).

Methods

Sample collection

A large area of rice is harvested from late September to late December in ricefields of Sevilla province within the Guadalquivir delta. On three separate days in November-December 2014 and 2015, we collected 13 adults of *Procambarus clarkii* that were crawling out at the edge of ricefields that were being harvested, as well as gull faeces and pellets from different locations (Figs. 1, 2, 3; Table S1). We put crayfish immediately into individual plastic jars (12cm high, 9cm diameter) filled with deionized water for 10 minutes to wash off propagules attached to the outside of the animals. Crayfish were then placed in a cool box on ice in the field and frozen on arrival to the laboratory. Fresh samples of *L. fuscus* excreta (with a characteristic shiny appearance before air drying begins) were collected from large, monospecific flocks that were flushed while resting on the dykes separating individual fields. Samples were taken from points separated by >1 m and were likely to be from distinct individuals. Excreta were carefully inspected, removing any soil or gravel from the sample with tweezers or a knife before placement in a plastic zip-bag and storage at 4°C for up to 3 weeks until processing. Prior to processing, once removed from the fridge, each sample was again checked under the microscope to remove any seeds or eggs on the outside that potentially had stuck on from soil or via wind).

Sample processing

Within 2 days, the contents of the plastic jars where crayfish had been washed were sieved through a 100 µm sieve and inspected in petri dishes under a stereomicroscope, to search for plant diaspores (angiosperm seeds and charophyte oogonia) and invertebrates or their eggs. Gull samples were weighed then processed in a similar way, washing them on the sieve with deionized water. The mass of freshly collected *L. fuscus* faecal samples (n = 76) was 1.8 g ± 0.30 (mean ± s.e., range 0.21-6.67 g) and the mass of regurgitated pellets (n = 14) was 6.9 g ± 1.34 (range 3.82 – 12.79g). The frozen crayfish were defrosted and dissected, inspecting the contents of the whole digestive system under the stereomicroscope coupled with a digital camera.

Intact propagules were collected and counted. Propagules were photographed and measured via Axiovision software, then stored within a fridge (4°C) in Eppendorf® tubes filled with deionized water. For seed identification, we inspected shape, size and seed coat pattern and compared these traits with available literature (Cappers, Bekker & Jans, 2012; Bojnanský & Fargašová, 2007; Talavera & Castroviejo, 1999; Benedí & Orell, 1992). For seeds whose identification was problematic, we compared them with token specimens held within the University of Seville Herbarium. Intact diaspores were later placed in petri dishes with moistened filter paper in germination chambers set at 12 h of light at 24 °C and 12h of darkness at 18 °C. Germination tests were run for 3 months, checking every day for new germinants, which we counted and removed from the petri-dishes.

Live invertebrates and eggs found in the samples obtained from washed crayfish were placed directly into 90% ethanol for later identification. Bryozoans were identified in Sevilla after Wood & Okamura (2005). Any remaining invertebrates and their eggs were sent to Valencia, where identifications were made following Alonso (1996), Dussart (1967), Einsle (1993), Hart & Hart (1974), Meisch (2000) and Karanovic (2012). Invertebrate eggs extracted from the excreta of gulls were then introduced in a 50 ml polypropylene aquarium with 40 ml of commercial mineral water (Cortes^{TM}), with the aim of stimulating hatching. Aquaria were placed in a culture chamber for one month at a constant temperature of 20ºC and a 12 h light: 12 h dark photoperiod.

Statistical analysis

Total numbers of propagules were compared between sample types (i.e. crayfish, faeces or pellets) using generalized linear models (GLMs), with a negative binomial error distribution using the glm.nb function in the MASS package (Venables & Ripley, 2002) for the R stats package (version 3.3.2; R Core Team 2016). Sample type and sample year were fitted as categorical variables, and sample mass (log transformed) was included as a continous variable when comparing pellets with faeces (mass data were not available for mud washed from crayfish). At the level of individual propagule taxa, we were unable to develop satisfactory GLMs due to the dominance of zero values in the data, and problems of model convergence and overdispersion. Because pellets were expected to contain larger items than faeces, we used a non-parametric Mann-Whitney test to compare the number of rice grains (including zero values) between pellet and faecal samples using the R stats package. Rice grains were much larger than other propagules recorded (Table 1). The germinability of diaspores recovered from gull excreta and from crayfish was compared for the most abundant taxon (toadrush *Juncus bufonius*) with a Fisher exact test.

The structure of the communities of plant and animal propagules present in different sample groups (faeces, pellets or washed crayfish) and years were compared with Permanova (i.e., permutational MANOVA; Anderson, 2001) using the adonis function of vegan in R. Only samples for which at least one propagule was recorded were used for Permanova, and data were Hellinger-transformed (Legendre & Legendre, 2012). One sample (which contained only one species *Streptocephalus torvicornis*, not found in any other sample) was excluded from further analyses in Permanova and other multivariate tests (see below). When differences between groups (i.e. sample types) were observed in Permanova, we checked which taxa contributed most to those differences using SIMPER (similarity percentage; Clarke, 1993, also implemented in vegan). Groups of samples were also analysed to test for multivariate homogeneity of group dispersions (Anderson 2006), with the function betadisper in vegan. This test is a multivariate analogue to Levene's test, and is related to a comparison of the beta-diversity observed for each group of samples. Non-metric multidimensional scaling (NMDS) was used to obtain an ordination of the community composition for the same sets of samples used in Permanova. NMDS was carried out using the metaNMDS function in vegan, and the Bray-Curtis distance was applied to calculate the distance matrix.

Results

The contents of pellets indicated the birds were feeding mainly on alien crayfish (*Procambarus clarkii*) and rice grains (*Oryza sativa*). Of 14 pellets examined, 13 were dominated by pieces of crayfish exoskeleton (Fig. 2).

Plant diaspores carried by crayfish

In total 175 plant diaspores from 11 taxa (Table 1) were attached externally to crayfish (mean ± s.e. = 13.46 ± 5.89 per crayfish; median = 6.0) and were washed off together with the mud coating the exoskeleton (Fig. 1). Diaspores were dominated by Juncaceae, 56 % being *Juncus bufonius* and 14 % *J. subnodulosus*. Overall, 19 % of the diaspores germinated, representing seven taxa (Table 2). Upon dissection of the 13 crayfish, only broken seeds (2 fragments of *Juncus bufonius*, one each of *Cyperus difformis*, *Juncus* sp., and *Polypogon* sp.) were recovered from the stomach contents, and no diaspores were recorded in the intestines.

Plant diaspores in gull excreta, and their comparison with crayfish

In total, 122 diaspores (mean ± s.e. = 1.61 ± 0.34 per sample; median = 1) were found in faecal samples and 49 (mean± s.e. = 3.50 ± 2.19; median = 0.5) in pellets (Table 1). These belonged to 11 plant taxa, but 63% of the diaspores were of *Juncus bufonius*. Pellets contained significantly more rice grains per sample than faeces (Mann-Whitney test, N = 76, 14, W = 602, $P = 0.013$). However, in a GLM of the total number of diaspores per sample, there was a positive partial effect of sample mass (N = 90, z = 2.872, $P = 0.004$), but no significant difference between pellets and faeces (z = -0.861, $P = 0.39$) or between years (z = 0.301, $P = 0.76$). When this GLM was repeated after removing rice grains, the partial effect of sample mass was retained and there were significantly more other diaspores in faeces than in pellets (z = 4.136, $P < 0.001$).

Germination was recorded for six plant taxa with 23% germinability overall for diaspores from faeces and no germination for pellets (Table 2). Few *J. bufonius* seeds were recovered from pellets (Table 1) and, although none germinated, the difference in germinability with those from faeces (18%) was not significant (Fisher Exact Test, $P = 0.60$).

There was a similarity in the observed species composition between the plant diaspores found on the outside of crayfish, and those found in gull excreta, especially in faeces (Table 1, Fig. 4a). Of 13 taxa recorded, nine were found in both crayfish and gull faeces, and *J. bufonius* was dominant in both. In a GLM comparing the total number of diaspores between sample types (including rice, without controlling for sample mass), there were significantly more diaspores in crayfish samples than both groups of gull excreta ($N = 103$, $z = 4.654$, $P < 0.001$) and more in pellets than faeces ($z = 2.123$, $P = 0.034$), whilst the year had no effect ($z = -1.140$, $P = 0.17$). When this analysis was repeated without rice grains, there remained significantly more diaspores on the crayfish than in excreta ($z = 5.250$, $P <0.001$), and there were now fewer diaspores in pellets than in faeces ($z = -2.203$, $P = 0.03$). There was no difference in the germinability of *J. bufonius* seeds recovered from gull faeces (18%) and from outside crayfish (22%, Fisher exact test, $P = 0.58$).

We found significant differences in the community composition of plant propagules between sample types (faeces, pellets or washed crayfish, $N = 42$, 7, 13 respectively, d.f. = 2, $F = 3.797$, $P = 0.001$), and between sampling years (d.f. = 1, $F = 3.119$, $P = 0.015$) when analysed with a Permanova, and these differences were not related to differences in multivariate dispersion, as there was a similar average distance to the median in both years (betadisper, d.f. =1, $F = 1.426$, $P = 0.237$) and in the three types of samples (d.f. = 2, $F = 0.487$, $P = 0.617$). The observed difference between years was due to changes in abundance for some species. In 2015 there were significantly more *R. sceleratus* and *J. subnodulosus* seeds than in 2014 (SIMPER, $P < 0.001$). Also, there were significantly more *J. bufonius* and *A. retroflexus* in faeces than in pellets, more *O. sativa* seeds in pellets (SIMPER, $P < 0.05$), and more *R. sceleratus* on crayfish than in gull excreta (SIMPER, $P < 0.001$).

Invertebrates carried by crayfish

In total, 213 living adult or juvenile invertebrates (mean ± s.e. = 16.23 ± 3.14 per crayfish; median = 18) from at least ten taxa were recorded in mud samples washed from crayfish

(Table 4). The most abundant invertebrates were the Copepoda, but the most diverse groups were the Cladocera (four species) and Ostracoda (at least three species). A total of 54 invertebrate propagules were also recovered from external surfaces of crayfish (mean ± s.e.= 4.15 ± 2.07 per crayfish; median = 2), representing at least eight taxa (Table 4). The most abundant propagules were anostracan eggs, cladoceran ephippia and ostracod eggs, with smaller numbers of bryozoan statoblasts (Table 4). No invertebrate eggs were recovered in the digestive system of the crayfish.

Invertebrates in gull excreta, and their comparison with crayfish

In total, 96 invertebrate propagules were recovered from gull faeces (mean ± s.e. = 1.26 ± 0.31 per sample; median = 0) and 33 (mean = 2.36 ± 0.83 per sample; median = 1) from pellets, representing 12 crustacean, bryozoan and annelid taxa, five of which were also recorded on crayfish (Table 3). The most abundant propagules were from Anostraca, Annelida, Cladocera and Ostracoda. Statoblasts of three *Plumatella* species (Bryozoa) were recorded, including *P. vaihiriae*, which is an alien species spreading across Europe (Taticchi, Pieroni & Elia, 2008). In a GLM of the total number of invertebrate propagules per excreta sample, there was no significant partial effect of sample mass ($N = 90$, $z = 1.210$, $P = 0.23$), nor a difference between pellets and faeces ($z = -0.624$, $P = 0.53$), but there were significantly more propagules in 2015 ($z = 2.50$, $P = 0.013$).

Unlike crayfish samples, live adult or juvenile invertebrates were not recorded from gull excreta (Table 3). Indeed, we found differences in community composition between crayfish samples, pellets and faeces (N = 9, 7, 31 respectively, Permanova, d.f. = 2, $F = 2.0642$, $P = 0.044$), owing mainly to the abundance of adult or juvenile *Eucyclops* and *Onychocamptus* copepods on crayfish (SIMPER, $P < 0.001$), and the greater abundance of *Artemia* type eggs (SIMPER, $P < 0.01$) and ostracod eggs ($P < 0.05$) in pellets (Table 3, Fig. 4b). These differences in community composition were also related to significantly wider multivariate dispersion (betadisper, d.f. = 2, $F = 6.718$, $P = 0.003$) in faeces (average distance to median = 0.61) compared to pellets (average distance = 0.43) and crayfish (average distance = 0.47), as the former contained a wider array of taxa (Table 3, Fig. 4b).

Discussion

We have shown *L. fuscus* may facilitate secondary-dispersal of plants and invertebrates, including agricultural weeds and exotic invertebrates (Tables 1, 3, 4). Apart from the much larger rice grains ingested as food items, the propagules we detected are small and inconspicuous and likely to be overlooked in conventional studies of avian diet. With the exception of adult and juvenile invertebrates, the propagules recorded in gull excreta and on crayfish are from similar taxa and often in similar proportions. Where differences were observed in community composition (e.g. Fig. 4b), this is likely to be explained by the greater sampling effort for gull excreta, which detected propagules from a greater number of invertebrate taxa. Our results suggest most of the propagules dispersed by gulls are ingested inadvertently when feeding on crayfish. This constitutes secondary dispersal, since the crayfish themselves were dispersing these propagules within mud adhering to their exoskeleton while moving within the ricefields. The daily movements of the gulls between fields (Bouten et al. 2013) are likely to greatly facilitate the spread of propagules across the 37,000 ha of ricefields in the Doñana area. Most of the propagules recorded in gull excreta will readily survive in the moist or dry fields until conditions become suitable for growth. For aquatic species, this will be after the reflooding and sowing of fields with rice in May. Many of the plants are more terrestrial, e.g. *A. retroflexus* or *S. vulgaris*, and can complete their life cycle before May.

On extreme occasions, *P. clarkii* have been known to disperse up to 4 km on land in one day, but they usually move <10 m per day (Anastacio et al., 2015). Over time, short distance dispersal (SDD) may lead to range extension through multiple SDD events (Coughlan, Stevens, Kelly, Dick & Jansen, 2017). Nevertheless, secondary dispersal by gulls will greatly increase the overall dispersal distance for most taxa whose propagules become attached to crayfish. Many of the taxa identified in gull excreta have not previously been recognized to have a capacity for avian zoochory. *Larus fuscus* has an increasing population of around 600,000 birds in western Europe and west Africa (Wetlands International, 2017). Therefore, given their regular movements within and beyond southern Spain (Bouten et al., 2013, Shamoun-Baranes et al., 2017), *L. fuscus* may facilitate LDD of plants and invertebrates, enabling their rapid spread over broad areas. Tracking of individual gulls shows they move between Doñana ricefields and other Andalusian wetlands including Doñana fish ponds and natural closed-basin lakes such as Fuente de Piedra in Malaga (see Bouten et al. 2013).

Plant dispersal by L. fuscus and P. clarkii

Most of the plants we recorded are agricultural weeds, and many of them are aliens (Table 1). Earlier studies have shown gulls to be vectors of other alien plants and weeds (Green, 2016), although such dispersal has not previously been linked to predation on other seed vectors such as crayfish. In the plant trait database Baseflore (Julve, 1998), only 4 of the 11 plant taxa found in gull excreta are considered to have zoochory dispersal syndromes (i.e. to be animal-dispersed), and none of them are assigned to the endozoochory syndrome because they all lack a fleshy fruit. The most abundant plant we recorded, *Juncus bufonius*, is assigned to the epizoochory syndrome, and Darwin (1872) germinated a seed removed from mud attached to the leg of a woodcock *Scolopax rusticola*. In particular, *J. bufonius* can be a highly abundant weed in agricultural fields sown with different crops across Europe (Devlaeminck, Bossuyt & Hermy, 2005), and when dispersed by birds it is often likely to be moved to suitable habitat.

Given the large number of *L. fuscus* present, and the high proportion of plant taxa recorded in small numbers (Table 1), it is likely that many other plant species are dispersed by gulls in Doñana ricefields (e.g. other ricefield weeds found in Spain, Kraehmer et al., 2016). On the other hand, *L. fuscus* is unlikely to be the only avian vector for the plant species we recorded. Other waterbirds feeding on crayfish in ricefields (Tablado et al., 2010) are likely to disperse these plant species, and a recent review of dabbling duck diet recorded four of the 11 species (Soons et al., 2016). Moreover, the migration routes of *L. fuscus* and other waterbirds feeding on crayfish in Spain (e.g. white stork *Ciconia ciconia*) extend into Africa (Shamoun-Baranes et al., 2017; Rotics et al., 2017). Accordingly, there is a potential for LDD of plants and invertebrates between continents.

European ricefields are affected by many weed species, some of which are alien species (Vasconcelos, Tavares, & Gaspar, 1999). All seven of the agricultural weeds we recorded (Table 1) are already known to have herbicide resistant populations (Heap, 2009). Herbicide resistant weeds reduce crop production and increase herbicide costs (Powles & Yu, 2010), and LDD of herbicide-resistant genotypes via waterbirds is likely to exacerbate these problems (Farmer et al., 2017). Furthermore, two of the plants recorded are alien to Europe (Table 1), and others are important aliens in other continents (Benedí & Orell, 1992; Pyšek et al., 2009). Migratory ducks have previously been found to act as primary dispersal vectors for alien plants and weed species in ricefields in France and the USA (Powers, Noble & Chabreck, 1978; Brochet, Guillemain, Fritz, Gauthier-Clerc, & Green, 2010).

We found that the largest seeds (rice grains) are more likely to be egested in pellets, as consistent with size selective treatment of food items in other waterbirds producing pellets (Sánchez, Green & Castellanos, 2005). We germinated seeds recovered from gull excreta for all species (except rice) for which more than seven seeds were recovered (Table 2). This suggests that, as for Anatidae (Green et al., 2016), small seeds from any plant taxon are likely to survive passage through the alimentary canal of gulls. Moreover, a study examining endozoochory by yellow-legged gull *L. michahellis* suggests that seeds egested in faeces are retained in the gut for longer (median 14 h for glass beads) than those in pellets (median 5 h; Nogales, et al., 2001). Faeces are also produced at any time of the day in a range of habitats, whereas pellets are more likely to be produced at roost sites.

Invertebrate dispersal by L. fuscus and P. clarkii

To our knowledge, this is the first study to report evidence of endozoochory of invertebrates by any gull species. Our observations of various microcrustacean groups and bryozoans in both dispersal vectors show that crayfish and gulls can increase propagule pressure of aquatic invertebrates at local scales, and also facilitate LDD. Our failure to hatch invertebrate propagules may be due to our protocol, which involved storing eggs in water in one lab for several months before posting to a second lab for identification and hatching. When fresh waterbird excreta are placed rapidly for hatching, many invertebrate taxa hatch (Frisch, Green & Figuerola, 2007; Brochet, Gauthier-Clerc et al., 2010; Valls et al., 2017).

We recorded a variety of ostracods, cladocerans and copepods as adults or juveniles on the external surface of *P. clarkii*, which is likely to be an important dispersal vector at a local scale (Ramalho & Anastácio 2015), and to enable the survival of microcrustaceans moved to water (e.g. to drainage canals) on crayfish when they would otherwise die when the ricefields are dried out. Ostracods recorded included the entocytherid *Ankylocythere sinuosa*, an alien of American origin which is commensal on *P. clarkii* in Europe (Aguilar-Alberola et al., 2012), and the cypridid *Hemicypris reticulata* (Klie, 1930), which has not previously been recorded in Europe. This latter species has been found in various biogeographical regions (including the Neotropical, Oriental and Paleotropical), usually in ricefields (Savatenalinton & Martens, 2008; Martens et al., 2013). It has been recorded in North Africa, which shares migratory waterbirds with our study area (Rendón et al., 2008). It is possible that *H. reticulata* is native to southern Spain but has been overlooked until now; however its wide distribution and the common occurrence of exotic ostracods in ricefields (Valls et al., 2014) suggest this is most probably a new alien which is potentially invasive in Europe. The abundance of ostracod eggs in gull excreta, plus previous evidence that ostracod eggs can survive gut passage by waterbirds (Brochet, Gauthier-Clerc et al., 2010; Rogers, 2014; Valls et al., 2017), suggest that these ostracods are secondarily dispersed by *L. fuscus*. This is less clear in the case of the *A. sinuosa*, which is not known to produce diapausing eggs (Mestre et al., 2013). However, adult ostracods can also survive gut passage through waterbirds (Green, Frisch, Michot, Allain & Barrow, 2013; Rogers, 2014).

We recorded adults or ephippia of eight cladoceran taxa, including six species previously reported from the Doñana area (Fahd et al., 2009) and which have an extensive geographical distribution. We also recorded anostracan eggs, which are known to be readily dispersed through the avian gut (Rogers, 2014), and may also survive passage through the gut of crayfish (Moore & Faust, 1972). We found eggs of tadpole shrimps *Triops* in gull faeces, which is consistent with previous records of their dispersal on the outside of *P. clarkii* (Pérez-Bote et al., 2005), and with genetic evidence suggesting a major role for avian vectors for *Triops* (Korn et al., 2010). In the case of copepods, we only recorded the presence of living animals on crayfish. However, we may have overlooked their small eggs in gull excreta. Equally, encysted adults may also be transported through the avian gut (Frisch et al., 2007). Both genera recorded (*Eucyclops* and *Onychocamptus*) are littoral/benthic and commonly associated with periphyton (Dussart, 1967), which grows on the carapace of crayfish. This is the first time the harpacticoid *Onychocamptus mohammed* has been reported in the Donaña region, but it has been previously reported from wetlands in Spain, Morocco and France (Aguesse & Marazanof, 1965; Alfonso & Miracle, 1990; Dakki, 1997; Martinoy et al. 2006).

Plumatellid bryozoans are frequently recorded in waterbird excreta (Brochet, Gauthier-Clerc et al., 2010; Green et al., 2013), but to our knowledge this is the first confirmation that birds are vectors for alien bryozoans such as *P. vaihiriae*, although this is considered likely for other aliens such as *Pectinatella magnifica* (Balounová, Pechoušková, Rajchard, Joza & Šinko, 2013). The ability of bryozoans to disperse by birds may increase the economic costs they impose through biofouling of pipes and pumps (Mant, Moggridge & Aldridge, 2013). It is likely that the *Plumatella* statoblasts we recorded became stuck onto *P. clarkii* via mud. Although *P. repens* colonies have been reported growing directly on other alien crayfish in Europe (Duris, Horká, Kristian & Kozák, 2006), this has not been observed with *P. clarkii*.

Unpredicted consequences of the P. clarkii invasion

Procambarus clarkii is now the world's most cosmopolitan freshwater crayfish, and its introduction has led to dramatic negative impacts on aquatic ecosystems, the plant and animal communities they contain, and the ecosystem services they provide (McLaughlan et al. 2014, Souty-Grosset et al., 2016). We have shown that *P. clarkii* invasion also leads to novel dispersal pathways for plants and invertebrates through interactions with its predators. This represents an additional impact of this alien species on ecosystem services, which has not previously been recognized (McLaughlan et al. 2014). Given that so many of the species dispersed by *L. fuscus* are agricultural weeds or alien invertebrates, this dispersal facilitation is often likely to represent an ecosystem disservice rather than a service. Furthermore, because gulls and other birds move regularly between ricefields and other wetlands, they are likely to disperse alien species into natural habitats, promoting the invasion of the latter by novel species. Novel habitats such as ricefields can thus have unexpected impacts on natural ecosystems as a consequence of such dispersal interactions. Other biological invasions have been shown to lead to novel dispersal interactions involving alien vectors and/or alien propagules, but our study reveals one of few known cases of secondary dispersal in which the primary vector is alien (Hämäläinen et al., 2017). To our knowledge, it also constitutes the first demonstration of secondary dispersal that involves epizoochory in the primary phase and endozoochory in the second.

This work illustrates how biological invasions can reshape dispersal interactions in an unforeseen way, creating new pathways with the potential to increase rates of SDD and LDD and the spread of both alien and native organisms, including herbicide resistant weeds. Further empirical research is vital to identify the taxa dispersed by migratory waterbirds such as gulls in both natural and artificial wetlands, since they cannot simply be predicted, e.g. from seed morphology. Once such dispersal interactions are identified, movement ecology approaches will allow us to quantify their implications for metacommunities, the connectivity between artificial and natural ecosystems, and the geographical range of vectored organisms.

Acknowledgments

Faeces and pellets and crayfish were collected under permit from the Junta de Andalucía (N 2014/31). We greatly appreciate the help of Claire Pernollet, Edina Malmos and Tim Wood. Sample processing and germination tests were initiated in the Aquatic Ecology Laboratory (LEA-EBD). This research was supported by project CGL2016-76067-P (Agencia Estatal de Investigación y Fondo Europeo de Desarrollo Regional AEI/FEDER, EU to AJG), OTKA K108992 Grant (to AMV) and the New National Excellence Program of the Ministry of Human Capacities ÚNKP-17-3-I-DE-385 (to ÁL-K).

Authors contribution

ÁLK and AJG conceived the ideas and designed methodology; ÁLK, MIS, LV and XA collected the data; ÁLK, FMJ and AJG analysed the data; ÁLK, AMV, FMJ and AJG led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Table 1. Details of intact plant diaspores found in gull excreta and on the outside of crayfish, including the number of samples in which each taxon was recorded, the total number of propagules for each taxon, and the maximum number of propagules recorded in a single sample.

			Gull Faeces (n=76)			Gull Pellets (n=14)			Crayfish (n=13)		
Plant family	Plant taxa	Length (mm)	N samples with taxon	N diaspores	Max per sample	N samples with taxon	N diaspores	Max per sample	N samples with taxon	N diaspores	Max per sample
Amaranthaceae	*Amaranthus retroflexus*[†, ‡]	1.09	1	2	2	1	2	2	1	1	1
Asteraceae	*Senecio vulgaris*[‡]	2.42	1	1	1	-	-	-	3	5	3
Asteraceae	*Unidentified*	0.78	-	-	-	-	-	-	2	2	1
Charophyceae	*Unidentified*	0.56	10	17	7	-	-	-	5	14	8
Cyperaceae	*Cyperus difformis*[‡]	0.62	1	1	1	-	-	-	5	9	3
Euphorbiaceae	*Chamaesyce humifusa*[†]	1.04	8	9	2	-	-	-	-	-	-
Juncaceae	*Juncus subnodulosus*	0.36	5	7	3	-	-	-	7	25	13
	Juncus bufonius[‡]	0.43	30	77	12	3	3	1	9	97	62
Poaceae	Rice (*Oryza sativa*)	8.35	1	2	2	2	43	29	1	1	1
	Polypogon monspeliensis[‡]	0.78	2	2	1	-	-	-	1	1	1
Polygonaceae	*Rumex dentatus*	2.4	1	1	1	-	-	-	-	-	-
Portulacaceae	*Portulaca oleracea*[‡]	0.68	-	-	-	-	-	-	3	3	1
Ranunculaceae	*Ranunculus sceleratus*[‡]	1.06	3	3	1	1	1	1	10	17	3
	Total		42	122	33	7	49	33	13	175	97

[†] species alien to Spain, [‡]species considered agricultural weeds according to Heap (2009) and Agroatlas (2005), http://agroatlas.ru.

Table 2. Germinations of plant diaspores found in gull faeces and on the outside of crayfish. No germination was recorded for diaspores from pellets.

		Gull Faeces (n=76)		Crayfish (n=13)	
Plant family	Plant taxa	N Diaspores	N Germinated	N Diaspores	N Germinated
Amaranthaceae	*Amaranthus retroflexus*[†,‡]	2	2	1	1
Asteraceae	*Senecio vulgaris*[‡]	1	-	5	4
	Unidentified	-	-	2	-
Charophyceae	Unidentified	17	2	14	1
Cyperaceae	*Cyperus difformis*[‡]	1	-	9	3
Euphorbiaceae	*Chamaesyce humifusa*[†]	9	5	-	-
Juncaceae	*Juncus subnodulosus*	7	-	25	2
	Juncus bufonius[‡]	77	17	97	23
Poaceae	Rice (*Oryza sativa*)	2	-	1	-
	Polypogon monspeliensis[‡]	2	-	1	-
Polygonaceae	*Rumex dentatus*	1	1	-	-
Portulacaceae	*Portulaca oleracea*[‡]	-	-	3	-
Ranunculaceae	*Ranunculus sceleratus*[‡]	3	1	17	1
	Total	122	28	175	35

[†] species alien to Spain, [‡]species considered agricultural weeds according to Heap (2009) and Agroatlas (2005), http://agroatlas.ru.

Table 3. Details of intact invertebrate eggs recovered from gull excreta and from mud attached to crayfish, including the number of samples in which each taxon was recorded, the total number of propagules for each taxon, and the maximum number of propagules recorded in a single sample.

		Gull Faeces (n=76)			Gull Pellet (n=14)			Crayfish (n=13)		
	Invertebrate taxa	N samples with taxon	N taxon	Max per sample	N samples with taxon	N taxon	Max per sample	N samples with taxon	N taxon	Max per sample
Anostraca	*Artemia* cf.[‡]	5	9	4	4	11	5	3	4	2
	Streptocephalus cf. *torvicornis*	1	1	1	-	-	-	-	-	-
	Unid. Anostracan egg	4	6	2	-	-	-	-	-	-
Cladocera	*Alona* sp.	8	8	1	1	1	1	1	1	1
	Daphnia (*Daphnia*) "*pulex* group"	-	-	-	-	-	-	3	6	3
	Leydigia acanthocercoides (ephippia)	-	-	-	-	-	-	5	11	7
	Moina sp.	7	8	2	1	2	2	2	3	2
	Ceriodaphnia cf. *quadrangula*	3	4	2	-	-	-	-	-	-
Conchostraca	*Triops* sp.	1	2	2	-	-	-	-	-	-
Ostracoda	Ostracoda	11	33	8	5	17	8	5	21	12
Bryozoa	*Plumatella emarginata*	-	-	-	2	2	1	2	4	3
	Plumatella fungosa	2	2	1	-	-	-	-	-	-
	Plumatella vaihiriae[†]	1	1	1	-	-	-	-	-	-
Annelida	Unidentified	7	22	8	-	-	-	-	-	-
Non. Det.	Unidentified eggs	-	-	-	-	-	-	2	4	2
Total		32	96	32	7	33	17	9	54	32

[†] species alien to Spain

[‡]Morphologically these appear to be *Artemia*, but from the habitat this seems unlikely (due to low salinity), suggesting they are an unidentified Anostracan.

Table 4. Details of adult or juvenile living invertebrates recovered from the mud attached to the crayfish, including the number of samples in which each taxon was recorded, the total number of propagules for each taxon, and the maximum number of propagules recorded in a single sample. No living adults or juveniles were found in gull faeces or pellets.

	Invertebrate taxa	N samples with taxon	N taxon	Max per sample
Cladocera	*Macrothrix hirsuticornis*	4	4	1
	Alona affinis	1	1	1
	Leydigia acanthocercoides	2	7	6
	Tretocephala ambigua	1	1	1
Copepoda	*Eucyclops* sp. juveniles	3	12	10
	Eucyclops cf. *serrulatus*	12	97	40
	Onychocamptus mohammed	8	48	14
Ostracoda	*Ilyocypris* sp. juveniles	4	11	6
	Ilyocypris gibba	2	3	2
	Cyprididae juvenil	1	1	1
	Hemicypris reticulata†	1	2	2
	Ankylocythere sinuosa†	5	19	12
Nematoda	Unidentified	3	5	3
Total		13	213	100

† species alien to Spain

Figures.

Figure 1. Two crayfish *P. clarkii* at the edge of a ricefield in the study area. The upper specimen is coated in mud, whereas the lower specimen is cleaner owing to the effect of the water in its burrow. Credit Andy J. Green.

Figure 2. Fresh pellet regurgitated by a *Larus fuscus* in the study area, full of pieces of *P. clarkii* exoskeleton. Credit Andy J. Green.

Figure 3. Location of the study area and of sampling points in the ricefields on the west side of the River Guadalquivir. Numbers refer to sample sizes. The town of Isla Mayor lies in the middle of the points. Based on an image from Google Earth.

Figure 4. Non-metric multidimensional scaling (NMDS) plot showing the relation between propagules from mud washed off crayfish, and from the pellets and faeces of *Larus fuscus*, a) for plant propagules, b) for invertebrate propagules.

Supporting information

Table S1. Time and location of sampling of gull (*Larus fuscus*) excreta and moving crayfish (*P. clarkii*).

Collection date	Collection site	Sample size	Sample type
2014.11.20	37.12164° N -6.13612° W	30	Faeces
2014.12.03	37.04086° N -6.25420° W	7	Pellets
2014.12.03	37.07747° N -6.20839° W	3	Pellets
2014.12.03	37.04086° N -6.25420° W	13	Faeces
2014.12.03	37.07747° N -6.20839° W	18	Faeces
2014.12.03	37.15096° N -6.13015° W	2	Faeces
2015.11.06	37.17386° N -6.13023° W	3	Pellets
2015.11.06	37.14949° N -6.18833° W	1	Pellets
2015.11.06	37.17386° N -6.13023° W	9	Faeces
2015.11.06	37.14949° N -6.18833° W	4	Faeces
2015.11.06	37.22161° N -6.13796° W	6	Crayfish
2015.11.06	37.21551° N -6.13181° W	7	Crayfish

Figure 1. Two crayfish P. clarkii at the edge of a ricefield in the study area. The upper specimen is coated in mud, whereas the lower specimen is cleaner owing to the effect of the water in its burrow. Credit Andy J. Green.

Figure 2. Fresh pellet regurgitated by a Larus fuscus in the study area, full of pieces of P. clarkii exoskeleton. Credit Andy J. Green.

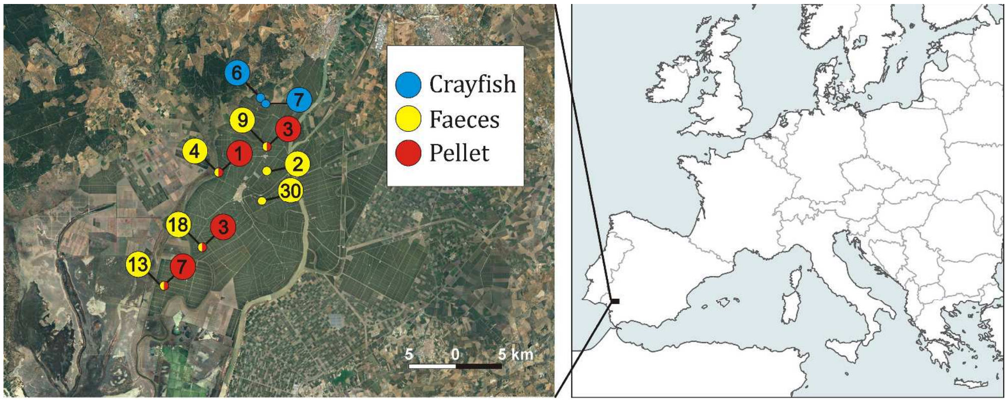
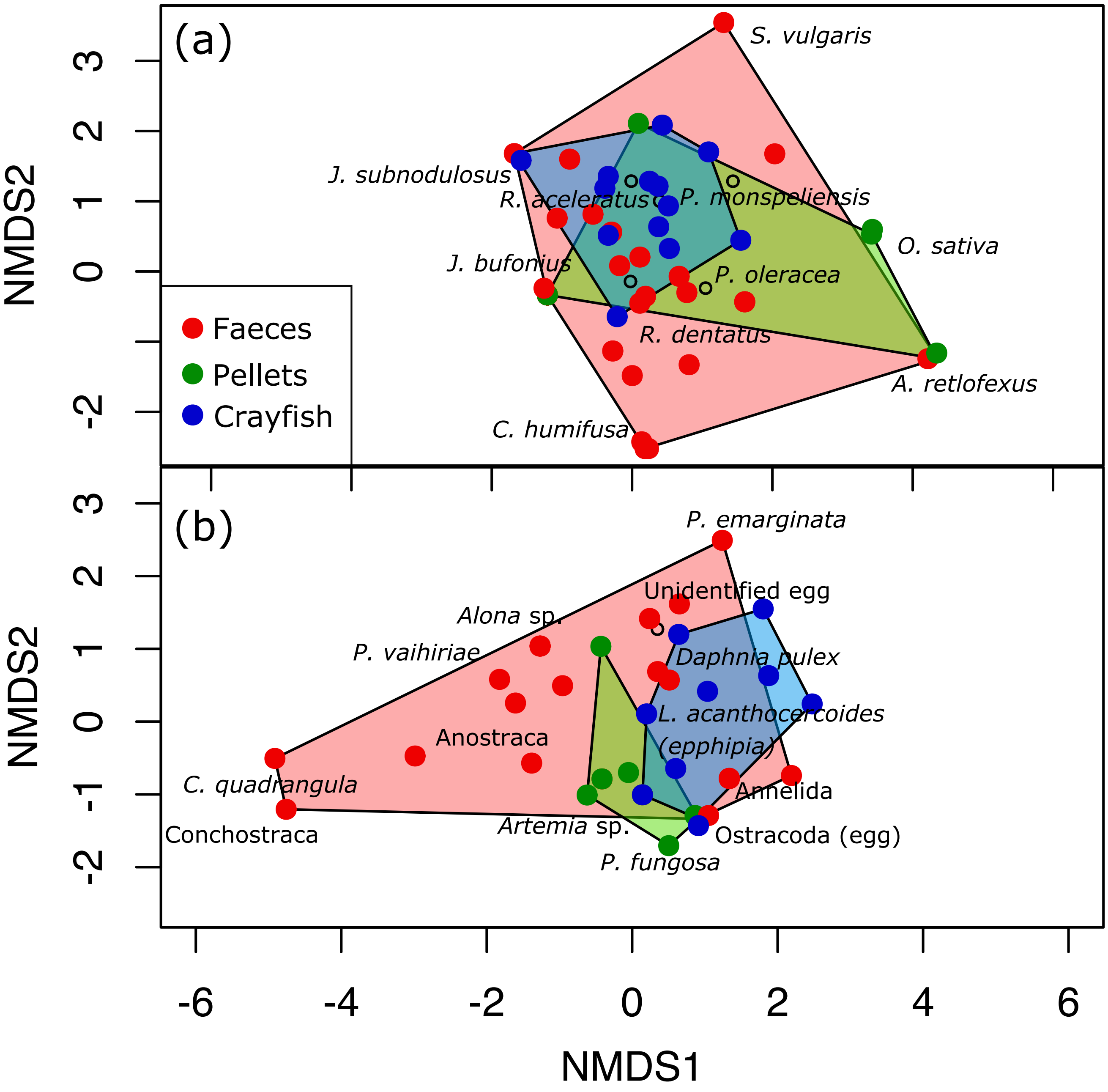


Figure 3. Location of the study area and of sampling points in the ricefields on the west side of the River Guadalquivir. Numbers refer to sample sizes. The town of Isla Mayor lies in the middle of the points. Based on an image from Google Earth.

997x393mm (96 x 96 DPI)


(a)
S. vulgaris
J. subnodulosus
R. aceleratus
P. monspeliensis
O. sativa
J. bufonius
P. oleracea
R. dentatus
A. retlofexus
C. humifusa
Faeces
Pellets
Crayfish
(b)
P. emarginata
Unidentified egg
Alona sp.
P. vaihiriae
Daphnia pulex
L. acanthocercoides
(epphipia)
Anostraca
C. quadrangula
Annelida
Conchostraca
Artemia sp.
Ostracoda (egg)
P. fungosa
NMDS2
NMDS2
NMDS1
3
2
1
0
-1
-2
-6
-4
-2
0
2
4
6