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Multiple seed dispersal modes of an invasive plant species on coastal dunes

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Abstract

Beach evening-primrose (*Oenothera drummondii* subsp. *drummondii*) is a perennial herb native to the coastal dunes of the Gulf of Mexico and southeastern USA. During the last century, the species was unintentionally introduced into coastal dune systems around the world. The purpose of this study was to explore the means of dispersal of this invasive in the newly established populations on the coastal dunes of Spain and to determine if they contribute significantly to the invasion process. After reconstructing the history of its spread in Spain from available records, we tested whether dispersion by marine currents can be a mechanism of colonization at regional scale, and whether the presence of native vertebrate participate in the expansion process at local scale. Through laboratory experiments, we demonstrated that the seeds of *O. drummondii* can be dispersed by seawater, since 0.63% present both buoyancy in seawater and subsequent germination after being washed with fresh water. This capacity for dispersal by marine currents could explain both the expansion of the species along the southwestern coast of Spain and the maintenance of its populations following disturbances such as storms. We also demonstrated that Iberian Hares and European Rabbits can disperse the seeds by endozoochory; seeds recovered from faecal pellets of these species were capable of germination (73 and 63%, respectively). *O. drummondii* has become an invader only in coastal dunes where these dispersers are present, and with no control actions to regulate their populations. In conclusion, *O. drummondii* is an alien species with varying dispersal methods, polychory. This helps maintain already established populations and disperses seeds at a regional level, depending on the incidence of storms and the presence or absence of rabbits and hares for efficient local seed dispersion. Thus, management actions that restrict marine and animal dispersal likely have the benefit of limiting the invasive potential of *O. drummondii.*

Keywords coastal dunes; endozoochory; *Lepus granatensis*; *Oryctolagus cuniculus; Spain; thalassochory*

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INTRODUCTION

Dispersion is a key process that determines the distribution patterns of species at local, regional and global scales (Auffret et al. 2014). Knowledge of the dispersion mechanisms of species is fundamental to understanding not only the natural processes of colonization and expansion, but also how biological invasions take place (Shigesada and Kawasaki 1997; Higgins and Richardson 1999; Silvertown & Charlesworth 2001). This can make a considerable contribution to early detection and control of invasive species (Puth and Post 2005; Hulme 2015).

Biological invasions begin with processes of long-distance dispersal (LDD), brought about either directly or indirectly by human action (Nathan et al 2008). Many different processes facilitate the human-assisted introduction of alien species, but international trade has contributed most to the dispersion of species beyond their natural range (Chapman et al. 2017). The routes of invasion and modes of transport are very diverse and at times complex, but remain little known for many species, especially those that have been unintentionally introduced (Essl et al. 2015). Nevertheless, this information is key to the establishment of effective invasion management strategies (Hulme et al 2008; Chapman et al 2017).

Once propagules arrive via LDD, successful establishment of new populations depends on: 1) the availability of suitable habitats, “climate matching” (Sutherst 2003) and “environmental matching” (Ricciardi et al. 2013), 2) the frequency of dispersion events (Drake & Lodge 2006), 3) the capacity of the populations to recolonize the zone following removal by stochastic events (Drury et al. 2007) and 4) the capacity of the species to integrate into the network of interactions of the resident ecosystem through the establishment of mutualist relationships and avoiding or overcoming biotic resistance (Richardson et al. 2000a; Levine et al. 2004; Traveset and Richardson 2014).

Introduced plant species that form self-sustainable naturalized populations are potentially invasive (Richardson and Pysek 2012), and transition to the status of invasive species largely depends on the reproductive efficiency and capacity for dispersion of the propagules that act to allow colonization and expansion from the initial points of introduction of the species in the territory (Richardson et al 2000b). The capacity of the propagules of alien plant species to invade natural habitats is greatly enhanced by adaptations to transport by animals, i.e., fleshy fruits (Richardson et al 2000a). However, even in the absence of conspicuous morphological adaptations of fruits or seeds, endozoochory can be an efficient mechanism for expansion of the alien species to new environments (Fernández and Sáiz 2007) even if this is not the manner in which it disperses in its native area.

Beach evening-primrose, *Oenothera drummondii* subsp. *drummondii* Hook. is a short-lived perennial herb in the family Onagraceae. This plant, which is native to the coastal dunes of the Gulf of Mexico and southeastern USA, is currently distributed in coastal dunes around the world (Dietrich and Wagner, 1988). Its introduction into non-native areas seems to be linked to the movement of goods in which the seeds of

O. drummondii were transported as stowaways (Frean et al. 1997; Heyligers 2008). Despite the fact that this species is considered an invasive in some countries or regions, there is little information available about it, for either native or non-native populations. However, recent studies show that, in Mediterranean coastal dunes, *O. drummondii* presents a higher competitive capacity than the native species (Zunzunegui et al. 2020). Interest in *O. drummondii* has been raised by the rapid expansion of the species observed in recent decades in some dune systems of southwestern Spain and the high impact it has on the diversity and functioning of the invaded dune systems (García de Lomas et al. 2015; Gallego-Fernández et al. 2019). Comprehensive knowledge of the different mechanisms of dispersion that can be utilized by an invasive species is rarely available, despite the interest in establishing effective measures of control (Gosper et al. 2005). With the objective of establishing a scientific basis for the management of this species, one of the first steps has been to identify the colonization and dispersion mechanisms employed by *O. drummondii* to maintain its non-native populations and to continue its expansion in zones where it is considered invasive.

While *O. drummondii* is an autochorous species and presents no evident adaptations for long-distance dispersion, two observations made by the authors suggest that this species could present various methods of dispersion: 1) in its native area of the Gulf of Mexico, the appearance of new populations has been observed in sectors of the coast in which the species was not previously recorded, which may suggest that the seeds reached these sectors via seawater dispersion, a mechanism characteristic of many species of beaches and dunes, and 2) partially consumed fruits have been observed in some non-native populations of Spain, suggesting possible dispersion by endozoochory. On the coasts of Spain, The Iberian hare and European rabbit are herbivores that disperse the seeds of a large number of native and non-native plants (Malo and Suárez 1995; Izhaki and Ne'eman 1997; Fernández and Sáiz 2007; Dellafiore et al. 2006, 2010), and thus they could be dispersers of *O. drummondii* seeds.

The purpose of this study was to understand the dispersal modes of *O. drummondii* in the introduced populations on the coastal dunes of Spain and to determine if they contribute significantly to the invasion process. First, we examined the available records of the distribution of the species on Spanish coastal dunes, which allowed us to reconstruct the history of its spread in the country. Afterwards, we tested whether dispersion by marine currents can be a mechanism of colonization of new areas by *O. drummondii*, as well as for the species recolonize areas after vegetation loss caused by the impact of storms. Finally, we studied how the species disperses in dune systems, and whether native vertebrate act as dispersers and promote the local expansion of the invasive plant. This information could help to explain the expansion of this species in the Gulf of Cadiz in Spain, and other coasts of the world, and to identify effective control methods in sites where it is considered invasive.

METHODS

The study species

Oenothera drummondii presents actinomorphic-herkogamous yellow flowers and a mixed mating system (self- and cross-pollination) with a high degree of self-compatibility both within its native distribution range and in non-native populations (Gallego-Fernández and García-Franco submitted). It is pollinated by hawkmoths in its native habitats (Wagner et al. 2007). The fruit is a dehiscent tetragonal capsule of 2-3 mm in width and 2.5-5 cm in length that splits longitudinally when wet, releasing numerous small seeds (*ca.* 0.6 x 1.2 mm) with no evident morphological adaptation for dispersal (Figure 1). In its native range, *O. drummondii* is relatively scarce and presents small populations (Moreno-Casasola, 1988; Gallego-Fernández and Martínez 2011). The authors have collected seeds from 17 populations along the Gulf of Mexico coast and have found no evidence of predation and/or biotic dispersion (by vertebrates). The species is considered invasive in Spain, Israel and China (Dufour-Dror 2012; Xu et al. 2012; Gallego-Fernández et al. 2019), but has also been introduced into Australia, South Africa, Egypt and New Zealand (ALA 2014; Shaltout et al. 2016; Frean et al. 1997; Heenan et al. 2002). The species has recorded near Spain, in Tetouan, Morocco, although it is not currently present (Hammada et al 2011), and in Marseillan, French Mediterranean coast, where the species has been present since 2014 (Tela Botanica, https://www.tela-botanica.org/). To date, however, studies have only been conducted on the effect of its invasion in the dunes of southern Spain, where it has caused considerable modification of the composition and abundance of species of the native plant community (Gallego-Fernández et al. 2019; Zunzunegui et al 2020).

The seeds of *O. drummondii* do not disperse far from plants. The capsules, once mature, open when environmental humidity is high and release the seeds, which fall to the ground beneath the mother plant (unpublished studies of JB Gallego-Fernández et al. of the seed bank of the species show that the majority of the dispersed seeds are found below the mother plant). This type of dispersion strategy is known as ombrohydrochory and is frequently found among other species of the genus *Oenothera* (Walck and Hidayati 2007). Secondarily, the small sized seeds can be dispersed by the wind to a distance of some metres from the parent (J.B. Gallego-Fernández, personal observation). This type of dispersion is easily observed in the foredune, where the incidence of wind is greater than in the dune zones further inland. At the end of winter, numerous seedlings are observed to the leeward of the parent individuals, extending up to 3-4 m in distance from the mother plant, although the seeds can be dispersed for greater distances (J.B. Gallego-Fernández, personal observation).

Species distribution in Spain

To determine the dates and locations of the first records of *O. drummondii* in Spain, we used data collected from the Global Biodiversity Information Facility (GBIF, http://www.gbif.org/), the Spanish Plants Information System (ANTHOS, http://www.anthos.es/), and the Herbarium of the University of Seville. Additional occurrence records for Spain were provided by the Regional Government of Andalusia and collected by field survey along the coast of Gulf of Cádiz and Cantabrian Sea. Subsequent visits were made to all of the dune systems in which the species has been recorded (Figure 1, Table 1). In each site, the population size was recorded, as well as the naturalized or invasive character of the population from the existing literature or through field observation. Populations were considered to be invasive when we observed a high number of individuals, widely distributed in the different habitats of the dune system (Blackburn et al 2011). In addition, the existence of any measure of control of the species and whether the dune system is found within a protected area were also recorded. Moreover, partial or total depredation of the fruits was registered, since this is an indication of the presence of potential seed dispersers. During previous studies (Gallego-Fernández et al 2019), depredated fruits were observed along with the presence of *O. drummondii* seedlings in the faeces of the Iberian hare (*Lepus granatensis* Rosenhauer) and European rabbit (*Oryctolagus cunniculus* L.). For this reason, evidence of traces of the hare and the rabbit in the study sites indicated the presence of possible dispersers.

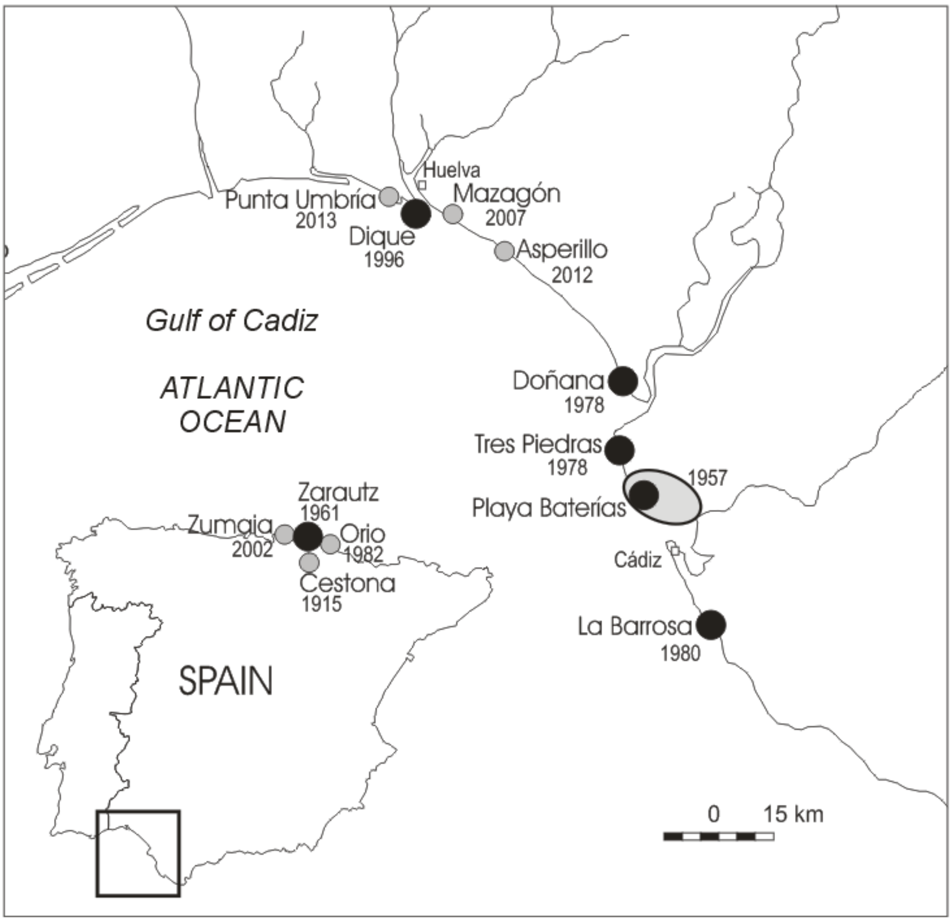

Fig. 1 Location of *Oenothera drummondii* subsp. *drummondii* Hook. in Spain based on Herbarium records and recent observations. Dates refer to the first record at that location. The area of the first introduction of the species in the Gulf of Cádiz appears as a grey oval. Grey circles denote the populations recorded, while black circles indicate the populations from which samples and/or fruits and faecal pellets were collected.

Dispersal by thalassochory

Seed survival in seawater

Germination tests were performed to determine whether seeds are capable of survival and germinate following immersion in seawater. The seeds used in this experiment were sourced from undamaged fruits collected in September 2015 in the Dique dunes. Previous germination experiments conducted with seeds from this locality and seeds from other populations of the Gulf of Cadiz showed similar germination patterns (results not shown in this study). The experiment consisted of eight treatments, placing the seeds in containers with 100 ml of seawater for 1, 3, 5, 7, 15, 30 and 60 days. In the control treatment the seeds were submerged in distilled water for 1 day, so that the seeds would have the same hydration as 1 day in seawater at the beginning of the germination experiment. The seawater used came from the same zone and had a salinity (total dissolved solids) of 31.3 ppt at 21 °C. Each immersion treatment included four batches (replicates) of 50 seeds each. The containers were maintained in the laboratory at ambient temperature and the water changed (where applicable) every seven days. Following the immersion period, the seeds were rinsed with deionised water. The lots of seeds were placed in Petri dishes of 90 mm in diameter on a double layer of filter paper moistened with 5 ml of water, and the dishes wrapped with Parafilm. These were left to germinate in a germination chamber with a regime of 12 h of light at a temperature of 20 ºC and 12 h of darkness at 15 ºC for 30 days. Seeds were inspected daily and those that had germinated were counted and removed. Seed germination was considered to have occurred when the radicle became visible.

When the seeds were in the seawater, it was observed that the majority sank while only a small proportion floated. Since buoyancy is very important for seed dispersion by marine currents (Guja et al. 2010), an additional experiment was conducted in order to determine the percentage of seeds that can float and their capacity for subsequent germination. Twenty lots of 100 seeds were placed in individual containers with 100 ml of seawater obtained in the Dique's zone. Every two days, for a period of 8 days, the number of seeds that floated after agitating the sample for 30 s was recorded. After the immersion period, the seeds were separated according to whether or not they floated, and all were rinsed with deionised water. Four replicates of 30 seeds that floated and four replicates of 30 seeds that did not float were allowed to germinate for 30 days, following the same protocol used in the previous germination experiments.

Salinity level effect and seed recovery

In order to determine the effect of different grades of salinity on seed germination, lots of 100 seeds were placed in Petri dishes on a double layer of filter paper and irrigated with 6 ml of water with eight salinity levels: 0 (control), 50, 100, 200, 300, 400, 500 and 600 mM NaCl. Four replicates per treatment were placed into a germination chamber for 30 days following the same protocol used in the previous germination experiments. A recovery experiment was then conducted in order to confirm the survival of seeds that did not germinate following initial incubation in NaCl. All of these seeds were rinsed in 100 ml deionised water and immediately transferred to Petri dishes lined with filter papers and irrigated with 5 ml deionised water. These seed germination assays were maintained for another 30 days under the same conditions as described above.

Dispersal by endozoochory

Seed production and fruit predation

The production of seeds and intensity of predation of the fruits of *O. drummondii* were evaluated in six locations that currently have populations of the species (Figure 1). In each site, individuals of similar mean size were selected at random (*ca.* 40 cm in diameter and 40 cm in height, although the individuals of Playa Baterías were of smaller size, at *ca.* 20-30 cm in diameter and 30 cm in height) at the end of September, before the first rains of autumn. In each plant selected, all of the mature fruits were counted, recording those that had undergone depredation. We obtained the average of fruit predation per plant for each study population. The mean size of the fruits (length) and mean number of seeds per fruit (seed set) were obtained in each population from 50 randomly selected fruits.

Seeds in faecal pellets - collection and germination experiments

Based on the visits and recordings of the possible dispersers, the populations from Dique (hares) and Tres Piedras (rabbits) were selected in order to study local scale dispersion. Fresh faecal pellets from hares and rabbits were collected in September just before the rainy season, when predation of hares and rabbits on ripe fruit is highest. The spatial pattern of defecation of the two species is different; rabbits usually defecate in latrines (Delives-Mateos et al. 2008), while hare faeces are scattered (Carro and Soriguer 2010). For this reason, the faeces were collected in different ways. In Dique, 964 hare faecal pellets were collected at random in an area of 2 ha within the semi-stabilized interior dunes, where the density of *O. drummondii* was high at *ca*. 6-8 plants/m^2. In the dunes of Tres Piedras, the rabbit faecal pellets were collected from 50 latrines (10-20 faeces from each, n=863), distributed in an area of 2.5 ha in the active dunes, where the density of *O. drummondii* was 0.2-2 plants/m^2. The faecal pellets were air-dried in the laboratory and stored in paper envelopes until subsequent processing.

A subsample (n = 100) of randomly chosen faecal pellets was taken from those collected at each site and these pellets were individually examined for *O. drummondii* seeds using a binocular magnifier. The seeds of *O. drummondii* are easily detected due to their orange-brown colour, small size (*ca*. 1.2 x 0.6 mm) and elliptical shape, which contrast with the detritus contained in the faecal material. Due to the low frequency of appearance of the seeds in rabbit faecal pellets, the number of pellets examined was increased (to 800 pellets). The average number of seeds per rabbit and hare faecal pellet was calculated. Germination experiments were conducted using the seeds obtained from the hare and rabbit faeces, as well as seeds obtained manually from undamaged mature fruits of each site.

Germination experiments were performed in February-March of the following year, with four treatments: seeds from fruits and seeds from hare faecal pellets collected in Dique, and seeds from fruits and seeds from rabbit faecal pellets collected in Tres Piedras. Four replicates of 50 seeds of each treatment were allowed to germinate for a period of 30 days, following the same protocol used in the previous germination experiments.

Finally, considering that the hare faecal pellets are often on the beach and can be moved by the sea and marine currents, taking with them the *O. drummondii* seeds they contain, experiments of survival and seed germination following some time in the seawater were also conducted, following the same protocol used with the seeds derived from fruits.

Data analyses

All data were tested for normality with the Kolmogorov-Smirnov and Lilliefors methods prior to analysis. Due to their non-normal distributions, the differences in the number of fruits per plant, fruit size, number of seeds per fruit, number of predated fruits per plant, proportion of faeces with seeds and time to first germination of the salinity treatments were compared between sites using Kruskal–Wallis H tests and Mann–Whitney U tests. A set of ANOVAs were used to detect differences in the final accumulated percentage of germination within the different sets of treatments, and a t-student test was used to detect differences in the proportion of plants with signs of depredation among sampling sites. An arc-cosine transformation was applied to the final accumulated percentage of germination data before performing parametric statistical analyses. A step-down pair-wise posteriori comparison was based on the Ryan-Einot-Gabriel-Welsh F (REGWF) procedure based on the F test (Meyers et al 2017). All statistical analyses were performed with SPSS 25.0 (SPSS Corp., Chicago, Illinois, USA).

RESULTS

Introduction and expansion in Spain

The first record in northern Spain is from the year 1915 in Cestona, an inland location at 8 km from the coast (Silvestre 1980) (Figure 1; Table 1). There are no references regarding how the species arrived. The lack of subsequent records indicates that the abundance and distribution of the species in the coastal region had not been considered very important. It is not until 1961 that it is cited once again, this time in the coastal dune system of Zarautz (Aizpuru et al. 1996). The species was also recorded in 1982 on Orio beach (Aizpuru et al. 1996) and on the Zumaia dunes in 2002, both zones very close to Zarautz, at 3 and 7 km, respectively. Intensive samples of the coastal flora conducted in 2001 (Silvan and Campos 2002), and field samples taken in 2017 by the authors, indicated that the species is currently only found in the Zarautz dunes. In 2017, control of the population began in the form of manual removal.

Table 1. Sites in Spain where the presence of Oenothera drummondii has been recorded. For each site, the geographic location, date of first record, presence of possible disperser animals, population size, current status of the population, history of control actions and location of the population within a protected area were recorded

Location	Latitude Longitude	First record	Potential Seed dispersers	Actual Population	Current Status	Control management	Protected area
North							
Cestona	43°14’N; 02°15’W	1915	-	-	-	-	No
Zarautz	43°17’N; 02°09’W	1961	No	≈ 300	Naturalize	Yes	Yes
Orio	43°17’N; 02°07’W	1982	No	-	Does not exist	No	No
Zumaia	43°18’N; 02°14’W	2002	No	-	Does not exist	No	No
South west							
Playa Baterías	36°39’N; 06°24’W	1957	No	76	Naturalized	No	No
Tres Piedras	36°42’N, 06°25’W	1978	Rabbits	>10,000	Invasive	Yes	No
Doñana	36°49´N, 06°24´W	1978	Hares, rabbits, red and fallow	6	Eradicated 2015	Yes	Yes

La Barrosa	36°21’N, 06°10’W	1980	No	21	Naturalize	Yes	No
Dique	37°09’N; 06°54’W	1996	Hares	>4 Millions	Invasive	Yes	Yes
Mazagón	37°07’N; 06°48’W	2007	No	-	Does not exist	No	No
Asperillo	37°04’N; 06°41’W	2012	No	1	Eradicated 2012	Yes	Yes
Punta Umbría	37°10’N; 06°56’W	2013	Rabbits	4	Eradicated 2017	Yes	No

In southern Spain (Figure 1), the first record was in the year 1957 around the village of Rota (Silvestre 1980). Since then, it has been recorded in another seven coastal localities along the length of 120 km of the coast of the Gulf of Cádiz. The next records are from 1978 in the dunes of Tres Piedras and Doñana (Silvestre 1980) located at 10 and 25 km in distance along the coast, respectively, to the north of Rota. On the beach of La Barrosa, the species was recorded in 1980 at a site 35 km to the south of Rota. In 1996, *O. drummondii* was recorded for the first time in the dunes of Dique (García-Mora pers. comm.), located around 80 km from Rota and 55 km from Doñana. Around Dique, some isolated individuals were recorded in 2007 in Mazagón at a distance of 14 km (de las Heras 2007), in 2012 in Asperillo at 20 km (Consejería de Medio Ambiente 2013) and in 2013 in Punta Umbría at 1 km (García de Lomas et al. 2015).

Oenothera drummondii control programs involving extraction of the plants, conducted by the regional government, have reduced (La Barrosa) or even eradicated (Doñana, Punta Umbría and Asperillo) its presence (Table 1). In the case of Doñana, located in a National Park, rigorous control has been applied since the end of the 1990s, such that it is currently considered eradicated (Consejería de Medio Ambiente 2016). The last six plants that remained in Doñana were removed and used in this study. However, in the dunes of Dique and Tres Piedras, despite the control actions undertaken, the species is considered to present invasive behaviour (García de Lomas et al. 2015; Gallego-Fernández et al. 2019).

The first record of introduction to the Gulf of Cádiz, in Rota, does not provide exact information regarding its location. Currently, there are two small-sized populations of *O. drummondii* in this municipality; one in a highway ditch situated around 1.5 km from the coast with around 50 individuals and another on the coast at Playa Baterías, with *ca*. 76 plants situated on the edge of a small sandy cliff next to the beach, with evidence of occasional erosion by the marine swell.

Dispersal by thalassochory

Seed survival in seawater

Seed germination was affected by the duration of immersion in seawater ($F_{6,56}$=51.86, p<0.0001) and the origin of the seeds ($F_{1,56}$=113.28, p<0.0001) (Figure 2). The final accumulated germination of seeds from fruits remained relatively high (86-90%) and similar to the control lots until 15 days of immersion in seawater, but it subsequently decreased significantly to 67 ± 4.8% and 13 ± 4.8% after 30 and 60 days of immersion, respectively. In turn, the germination of seeds from hare pellets was slightly lower (although not significantly so) than the control, and gradually decreased until 7 days of exposure. After 15 days, germination was significantly lower than the control, until reaching 47.5 ± 4.4% and 1.5 ± 1% after 30 and 60 days (Figure 2), respectively. In all cases, germination of seeds from fruits was significantly higher than that observed in seeds from pellets.

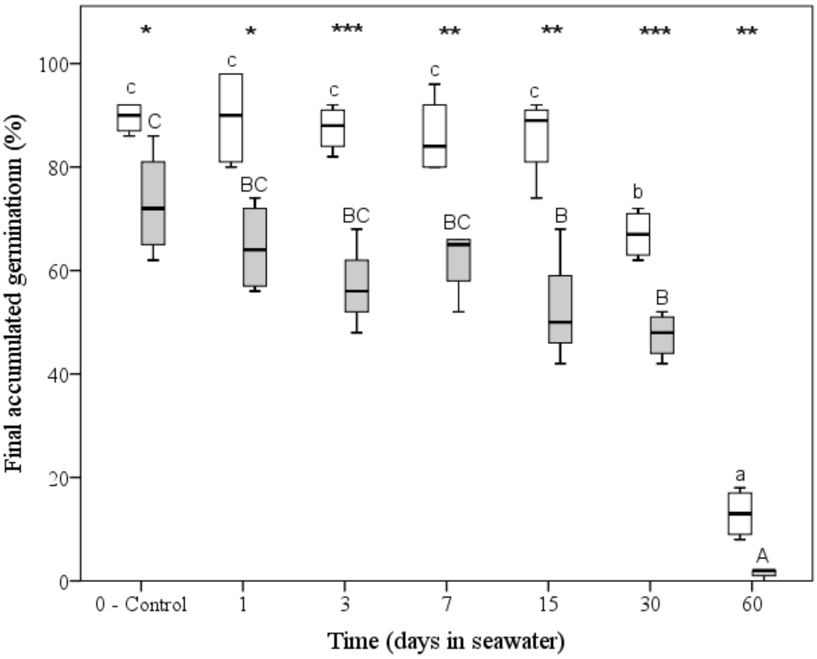

Fig. 2 Germination percentages of *O. drummondii* seeds obtained from plants in Dique (white boxes) and pellets from hares (grey boxes) after exposure to seawater for up 60 days. Lower case and capital letters indicate post hoc REGWF comparison tests between seeds from fruits and between seeds from pellets, respectively. Asterisks in the upper side indicate t-test comparison between the seeds from fruits and from pellets exposed for the same time period to seawater (* P<0.05; ** P<0.01; *** P<0.001). Each box shows the range between 25th and 75th percentiles; the central line shows the median. Vertical bars represent the highest and lowest values that are not outliers or extreme values

On the other hand, the buoyancy of the seeds decreased with duration of immersion in seawater (2 days: 9.8 ± 5.9% of floating seeds; 4 days: 7.1 ± 5.2%; 6 days: 6.2 ± 3.4%; 8 days: 5.4 ± 2.1%). The percentage of seeds that floated after eight days in seawater, 5.4 ± 2.1%, was significantly lower than those that did not float, 94.7 ± 2.1% (U= 5607, P<0.0001). Only 11.7 ± 4.3% of the seeds with buoyancy for eight days germinated after 30 days of incubation. This germination percentage was significantly lower than that of the seeds that do not float, 95.7 ± 5.7% (t=14.417, P=0.0001). Thus, around 0.63% of the seeds produced in the population have the capacity to float for eight days and subsequently germinate.

Germination response to salinity

Seed germination was negatively affected by increasing salinity levels (Figure 3). Seeds were only able to germinate at salinities of less than 200 mM, and only 4 seeds (1%) germinated at 200 mM. Significant differences in the effect of salinity on germination were observed between concentrations of 0 - 50 mM

NaCl (87.3 ± 2% and 86.8 ± 2%; respectively) and 100 mM NaCl (66.6 ± 2.2%) ($F_{2,9}$=31.51; P<0.001). Salinity significantly delayed the time of first germination ($H_{(2,12)}$=9.301; P<0.01); in the control concentration, and at 50 mM NaCl, this parameter was 4.5 ± 0.6 and 5 ± 0.0 days, respectively, and 6.8 ± 0.5 days in the 100 mM NaCl treatment.

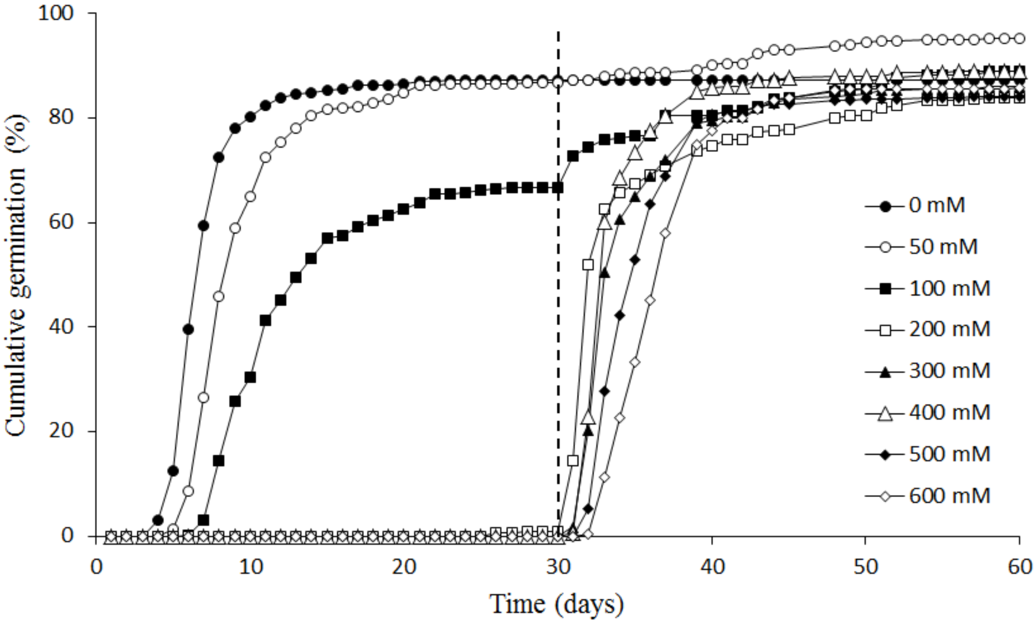

Fig. 3 Cumulative germination percentages of *O. drummondii* seeds exposed to a NaCl concentration gradient (0 to 600 mM NaCl). After 30 days, non-germinated seeds were rinsed in deionised water, and transferred to a new Petri dishes irrigated with deionised water in order to continue germination scoring. The vertical dashed line indicates the beginning of the recovery stage.

Following transfer to deionised water, germination of the seeds exposed to all salinity levels increased, except in the control (0 mM NaCl) where no new germination occurred until the end of the experiment. Although significant differences were found in accumulated germination among treatments ($F_{(7,24)}$=2.887, P<0.05), the accumulated germination after 60 days was very high (greater than 84%) in all of the treatments. It should be noted that the highest final germination was obtained in seeds that had been exposed to concentrations of 50 mM NaCl, 95.3%, however, germination in this latter treatment did not differ significantly from that of the seeds in the control treatment.

Dispersal by endozoochory

Fruit set and seed set

The mean number of fruits per plant differed significantly among sites ($H_{(5,157)}$=59.871, P=0.0001); the highest values were found in the plants of Tres Piedras and Doñana (Table 2). The size of the fruits and number of seeds per capsule differed significantly among sites (fruit length: $H_{(5,300)}$=196.996, P<0.0001; number of seeds: $H_{(5,300)}$=109.220, P<0.0001); the population of Dique presented larger fruits and higher numbers of seeds per fruit. These data indicate that the annual production of seeds per plant of the size selected in the studied populations varied from 6000 to 28000 seeds.

Fruit predation and seed dispersal

Predated fruits were only found on plants from Dique and Tres Piedras (Table 2), with the latter site presenting the highest proportion of plants with predated fruits, although both sites also presented plants with no evidence of fruit depredation. Equally, the mean percentage of depredated fruits was significantly higher (more than double) in Tres Piedras than in Dique (U = 371, P<0.0001). Predation was found from the apical zone of the fruit and it was observed, although not recorded, that most of the fruits were consumed for between half and two thirds of their length (Figure 4).

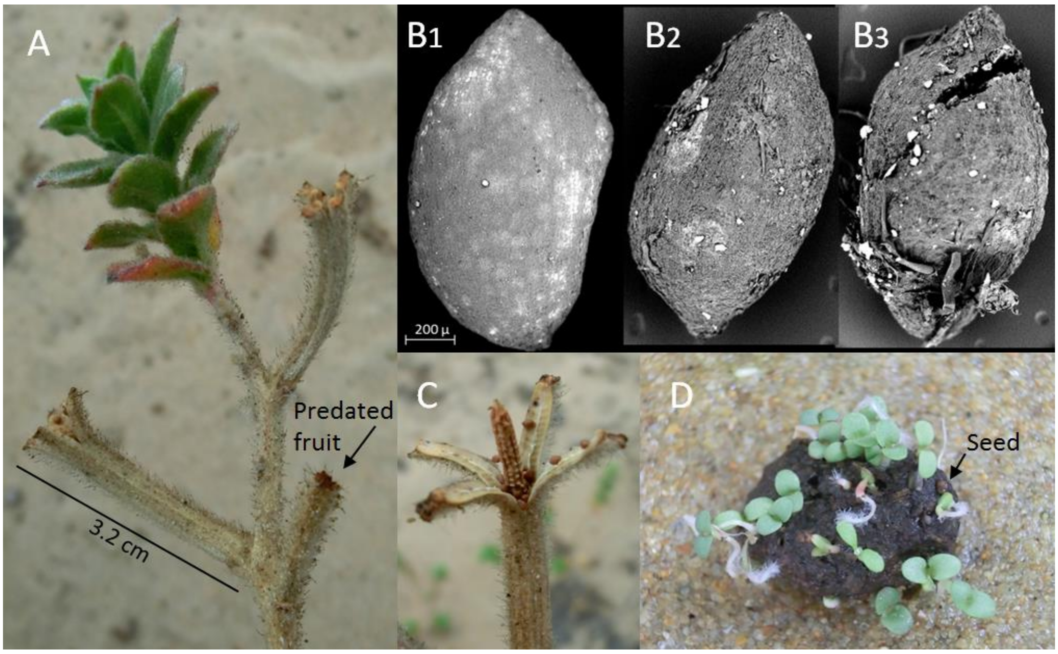

Fig. 4 (A) Branch of *O. drummondii* with intact fruits and fruit predated by hare, (B) EM photographs of (B1) intact seed, (B2) hare pellet seed, (B3) hare pellet damaged seed, (C) dehiscent fruit releasing seeds, (D) emergence of *O. drummondii* seedlings and seed presence in a hare faecal pellet.

Table 2. Number of fruits per plant (mean ± sd), percentage of plants with predates fruits, percentage of predate fruits per plant, fruits length and number of seeds per fruits of selected plants from 6 study sites. Comparisons between sites are indicated by small letters, same letters indicate non-significant differences (U-Mann-Whitney pair-wise comparison test after Kruskall-Wallis test).

Study sites	Plants	Number of	Plants with	Predated fruits	Fruit	Number of
Dique	40	105.2 ± 39.8 ^{b}	70.6	7.3 ± 8.0	34.2 ± 3.1 ^{a}	271.2 ± 49.9 ^{a}
Tres Piedras	40	131.4 ± 46.2 ^{a}	93.3	16.7 ± 10.9	31.9 ± 3.1 ^{b}	169.9 ± 62.8 ^{b}
Doñana	6	132.2 ± 23.7 ^{a}	0	-	21.3 ± 2.1 ^{e}	145.9 ± 29.1 ^{c}
Barrosa	21	99.9 ± 31.2 ^{b}	0	-	26.1 ± 2.6 ^{d}	155.2 ± 38.4 ^{bc}
Playa Baterías	20	36.3 ± 16.7 ^{c}	0	-	31.5 ± 3.5 ^{b}	171.18 ± 62.3

Zarautz	30	100.7 ± 27.5^{b}	0	-	29.7 ± 2.8^{c}	180.6 ± 37.4^{b}

The proportion of faeces with seeds was significantly higher in the hare faeces (94.0%) than in those of the rabbits (16.9%) (U=8674, P<0.0001) (Table 3). The mean number of seeds per faecal pellet was significantly greater in the hare faeces (U=3057; P<0.0001), with a maximum value found of 121 seeds in one hare faecal pellet, while the maximum number of seeds found in a rabbit faecal pellet was nine. The weight of the hare faecal pellets was significantly greater than that of the rabbit (U=974, P<0.0001). No correlation was found between the weight of either the hare or rabbit faecal pellets and the number of seeds present within (both P>0.05).

Table 3. Pellets weight, percentage of pellets with seeds, total number of seeds recovered and number of seeds per pellet from two dispersers of O. drummondii, European rabbits and Iberian hares.

Disperser species	Number of pellets	Pellets weight (g)	Pellets with seeds (%)	Total seeds recovered	Number of seeds per pellet (range)
Hare	100	0.215 ± 0.031	94.0	3142	31.42 ± 25.13 (0 - 121)
Rabbit	758	0.139 ± 0.133	16.9	227	0.30 ± 0.93 (0 - 9)

The percentage of accumulated germination after 30 days presented significant differences among treatments ($F_{(3,12)}=12.827$, P<0.0001) (Figure 5). The seeds derived from fruits of the two study sites presented a final accumulated germination (mean ± SD. Dique: 89.5 ± 3.0; Tres Piedras: 89.3 ± 5.9) that was significantly greater than those derived from faecal pellets of the hares and rabbits (73.0 ± 10.4 and 63.3 ± 2.9, respectively). The percentage of germination did not differ among the seeds from fruits, nor among those extracted from pellets.

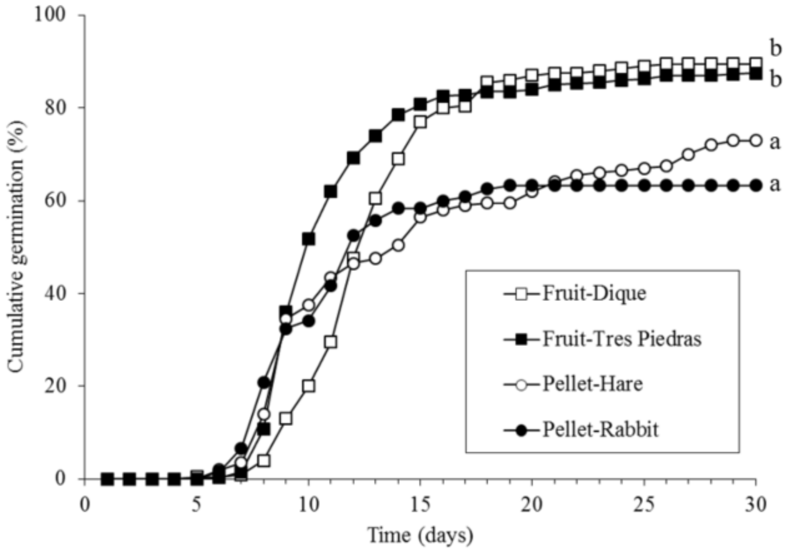

Fig. 5 Germination percentages of *O. drummondii* seeds obtained from plants and pellets of hares and rabbits at the populations of Dique and Tres Piedras. Different letters indicate differences between groups ($P < 0.05$) after the post hoc REGWF test.

DISCUSSION

At global scale, it is possible that the introduction of the species from its native areas is as proposed by Heyligers (2008) for populations in Australia: as stowaways in the ground-ballast of merchant shipping transporting coal from Texas in the first half of the 20th century. Heyligers (2008) also proposed that the subsequent expansion of the species along the eastern and western coasts of Australia was probably due to processes of natural dispersion and to transport by ship among the ports of Australia. To date, we do not know how the species arrived to Spain but it could have been by the same route. The main finding of this study is that the alien plant *O. drummondii* can use different methods of dispersion, polychory, that help maintain or expand its populations. At regional scale, and based on the results obtained in this study, the expansion of the alien species along the coasts of Spain could have been the result of specific dispersal events by means of storms and marine currents. Furthermore, the presence of disperser animals may be the determinant factor in the invasive behaviour at a local scale (Figure 6).

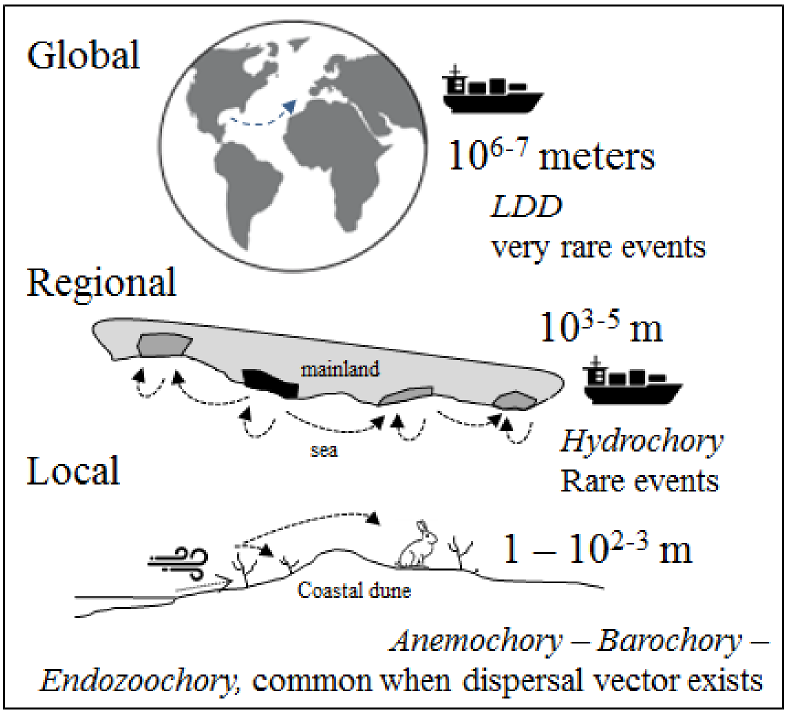

Fig. 6 Scale dependence of *O. drummondii* dispersal between dune systems. At global scales, dispersal between disconnected sites (i.e. native-introduced) requires long distance movement over ocean barriers (arrow dashed line). In a regional dune landscape, disconnected dune systems require medium-long distance transport by sea currents, with recolonization also produced following erosion by storms. At the local scale, wind can move the seeds to suitable areas, barochory consolidates populations and mutualism with dispersal mammals may spread the population across the dune system.

Seawater dispersal

In this study, we have demonstrated that *O. drummondii* has possibly dispersed via marine currents (thalassochory), since i) at least some of the seeds produced by plants of the Dique population can float in seawater and maintain their capacity for germination, and ii) once they arrive to beaches and dunes, germination can occur if the environmental variables (soil salinity) are favourable (Ridley 1930; Maun 2009). These attributes could have allowed the expansion of the species, via long distance dispersal (LDD) on marine currents from the zone of first introduction on the coast of País Vasco (Zarautz) and on the Gulf of Cádiz (Rota), to the other dune systems currently inhabited by the species or where it has been recorded for the last 60 years (Figure 1). These dispersion and germination characteristics can also allow a given population to be maintained on the beaches and dunes of a colonized site following disturbances brought about by the impact of storms (Gallego-Fernández et al 2020), which would in this case represent short-distance dispersal (SDD). This study also demonstrated the buoyancy of *O. drummondii* seeds for the first time. While most of the seeds did not float, 5.4% can float for at least eight days. The buoyancy of the seeds will surely decrease over longer periods of time since, as indicated by Guja et al (2014), the seeds will sink with the increase in their weight due to imbibition of the water. Marine dispersion is a non-standard mechanism of dispersal (Higgins 2003) of *O. drummondii*, since its seeds do not present morphological dispersal syndromes related to seawater dispersal. The species also complies with the second condition for dispersal via seawater, in that the seeds can maintain their capacity for germination following immersion in seawater even after 60 days, which is not the case in other terrestrial plants (Colmer and Flowers 2008; Maun 2009; Guja et al 2010).

Dispersion by seawater is characteristic of species that inhabit the supratidal zones and foredunes (Andersen 1993; Maun 2009), since it allows them not only to disperse over long distances, but also to survive in a habitat with unpredictable environmental conditions, facilitating the process of recolonization (Hesp 1991; Maun 2009; Guja et al 2010; Yang et al 2012). Dispersion by seawater can occur occasionally, as is the case with species of the supratidal zone, or, in species of the high beach and foredune, dispersion may occur very occasionally with storm surges that take place with frequencies that vary from various per year to once in a decade (Yang et al 2012). In the case of *O. drummondii* individuals that can grow on the high beach and foredune, the swell caused by storms, in addition to being capable of damaging the plants and eliminating the population, can remove the seed bank present in the sand and deposit them in the sea with erosion of these zones (Gallego-Fernández et al. 2020). The buoyant seeds can then be taken away by the marine currents or returned to the coast, enabling reestablishment of the population after the storms (Maun 2009; Yang et al 2012). In a simulation experiment conducted on the coasts of Shandong Peninsula, northern China, Yang (2012) showed that 90% of simulated seeds with the capacity to float returned the same sector of the coastline from which they came, while 1% of the simulated seeds that did not float were recaptured in the same sector of the coast. The *O. drummondii* seeds that cannot float (the majority) could present this type of local dispersion, where the action of the surf can return them to the shoreline together with the sediment where they can reach favourable zones, while the seeds that float can have this same destiny or undergo journeys of dispersion over greater distances, transported by marine currents.

Dispersion of propagules by waves and ocean currents has been described as a key factor in the rapid expansion of species that invade beaches and dunes over long distances (Heyligers 2007; Konlechner et al 2016). Dispersion can be as seeds, such as the case of *Cakile marítima* and *C. edentula* over thousands of kilometres of coasts of Australia and California (Barbour and Rodman 1970; Rodman 1986) and *Euphorbia paralias* on the coasts of Australia (Heyligers 2002), or seeds and fragments of plants, as occurs with *Ammophila arenaria* on the coasts of California, Australia and New Zealand (Buell et al. 1995; Konlechner and Hilton 2009). The capacity for dispersion by seawater can also maintain invasive populations, enabling them to overcome both natural disturbances and management actions intended to control them (Buell 1995; Hilton et al. 2005; Heyligers 2007; Rodman 1986).

On the other hand, the results of our experiments have shown that *O. drummondii* seeds are tolerant to salinity, even when exposed to concentrations equivalent to that of seawater (600 mM NaCl), and that their germination at relatively low saline concentrations levels is similar to when exposed to non-saline water. This response pattern has been described in other dune species worldwide (Maun 2009). If the seeds of *O. drummondii*, floating or otherwise, are deposited by the waves on the driftline of the beaches, germination will be impeded by the high salinity. However, since they are of small size, they can be lifted and carried by the wind (Maun 2009) towards the back beach, foredunes and even inland dunes. Moreover, on occasions, a high storm surge may transport seeds inland (Yang et al 2012). In such cases, the seeds can reach "safe sites", with soils of low salinity, where they can be washed by the rain and begin germination.

Endozoochory, the key to invasion

This study has demonstrated that the seeds of *O. drummondii* are dispersed through endozoochory by Iberian Hares and European rabbits. Both of these herbivorous species are recognized as seed-dispersal agents (Pakeman et al. 2003; Dellafiore et al. 2010, Mancilla-Leytón et al. 2013) that can contribute to the expansion of alien species (Traveset and Richardson 2006; Fernández and Sainz 2007; Calvino-Cancela 2011), although the role of the hare has been little studied to date.

Iberian Hares and European rabbits are considered foragers, and ingestion of seeds is accidental according to the hypothesis of "the foliage is the fruit" (Janzen 1984), and they thus act as non-specific dispersers of small and dry seeds (Malo et al., 1995; Izhaki and Ne'eman 1997; Fernández and Sáiz 2007; Dellafiore et al. 2010). However, in the case of *O. drummondii*, these two dispersers act as frugivores, since they consume the fruits of the plant directly (Figure 4), acting as specific dispersers (legitimate dispersers), despite the fruits and seeds of *O. drummondii* have no specific traits for dispersion by animals, for which reason this is also a case of a non-standard dispersal mechanism (Higgins 2003).

Although the predation of fruits of *O. drummondii* is low (Dique 7.3%, Tres Piedras 16.7%), the effectiveness of the dispersion (Malo and Suárez 1998) is very high, since: i) the quantity of seeds ingested is very high, ii) 63-73% of the seeds that pass through the digestive system of either frugivore germinate, which is a high level of survival of seeds following consumption by generalist herbivores (Mouissie et al. 2004), and iii) the seed dispersion carried out by each disperser is high, although it does differ according to species (31.4 and 0.3 seeds per hare and rabbit faecal pellet, respectively). Indeed, if we consider that each hare produces around 200 faecal pellets per day (Langbein et al 1999) and each rabbit produces 373 faecal pellets per day (González-Redondo 2009), one hare can disperse around 6280 seeds per day (of which, 73% can germinate) while a rabbit disperses around 112 seeds per day (of which, 63% germinate).

It should be noted that the only two sites where the species presents invasive behaviour are those in which the disperser animals of their seeds are present: Dique and Tres Piedras. The mutualist interaction established between *O. drummondii* and the native dispersers must play an important role in the expansion of this alien species, since it provides a complementary mechanism of short-distance dispersal, allowing the plant to colonize new sites, contributing to its local expansion (Nathan and Muller-Landau 2000) and facilitating the process of invasion (Richardson and Pysek 2012). The current distribution of *O. drummondii* in the coastal zones of Spain following its introduction *ca.* 60 years ago, is due not only to its great reproductive success, but also to the different mechanisms of seed dispersion it has been able to establish in its sites of colonization, where it presents relationships of frugivory with the local fauna.

Conclusions

Our study has shown that *O. drummondii* has been able to establish in several dune systems in the north and southwest of Spain since the middle of the 20th century. Regional expansion from the original point of introduction may have been caused by seed dispersal through ocean currents, as our experiments showed that some of the seeds can float in seawater and maintain the ability to germinate. In most of the dune systems where the species has been recorded, it has been maintained in small naturalized populations, some of which have been eradicated through early and sustained management. However, in the Dique and Tres Piedras populations, the species has an invasive behaviour. This is possibly because here, the seeds of *O. drummondii* are locally dispersed by endozoochory, by the Iberian hare and the European rabbit.

At regional scale, we recommend management measures to prevent the arrival of the species to coastal areas near existing populations. Management efforts should focus on beaches and foredunes to prevent the establishment of germinated storm-dispersed seeds. At a local level, in dunes where the rabbits and hares are present, we recommend management measures to contain the invasive species in the invaded areas by eliminating foci of invasion resulting from endozoochory. Once dispersal processes are known, both regional and local management can be coordinated to control and eradicate O. *drummondii* in the invaded dune area.

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