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Emerging invasion threat of the liana *Celastrus orbiculatus* (Celastraceae) in Europe

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Abstract

The woody vine *Celastrus orbiculatus* (Celastraceae), Oriental bittersweet, is an alien species that recently has been found to be spreading in Europe. Many aspects of its biology and ecology are still obscure. This study evaluates the distribution and habitats, as well as size and age of stands of *C. orbiculatus* in Lithuania. We investigated whether meteorological factors affect radial stem increments and determined seedling recruitment in order to judge the plant's potential for further spread in Europe. We studied the flower gender of *C. orbiculatus* in four populations in Lithuania and found that all sampled individuals were monoecious, although with dominant either functionally female or male flowers. Dendrochronological methods enabled us to reveal the approximate time of the first establishment of populations of *C. orbiculatus* in Lithuania. The youngest recorded individual with fruits was determined to be 10 years old. Analysis of radial increments revealed no reliable correlations with meteorological conditions. Therefore, we conclude that climatic conditions in the region are favourable for the growth, reproduction, and invasion of this species. *C. orbiculatus* produces viable seeds, successfully reproduces and spreads within and around the established stands. The presence of seedlings and two- to four-year-old saplings in the population confirms constant generative recruitment. Available information on the distribution of *C. orbiculatus* in Europe revealed its existence in 13 countries. In total, 58 occurrences of this species have been recorded in Europe so far. We consider that the lag period lasted until 2005 and that the exponential population growth phase has now set in. In Lithuania, the invaded area is quite small (0.51 ha); however, the total estimated invaded area in Europe could be about 250 ha. At the current stage of invasion and distribution in Europe, measures for control, management, and eradication of *C. orbiculatus* have a chance of being effective and economically feasible.

Keywords

dendrochronology, dioecious plants, distribution, flower gender, habitats, reproduction, seedlings

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Introduction

Lianas are strong competitors with trees for above- and belowground resources (Toledo-Aceves and Swaine 2008; Ladwig and Meiners 2010; French et al. 2017). Although vines and climbers are prevalent in gaps of tree stands and on forest edges where they compete intensely with trees, most of them are shade tolerant and can wait for favourable light conditions for a longer period of time (Baars and Kelly 1996; Schnitzer et al. 2000; Allen et al. 2007; French et al. 2017). When favourable conditions set in, lianas respond with high growth rates, reducing growth and recruitment of trees and shrubs (Greenberg et al. 2001; Leicht and Silander 2006; Ladwig and Meiners 2010). Thus, the impact of native, and particularly of invasive, lianas on biodiversity, habitats, and economically important trees make them a matter of serious ecological concern (McNab and Meeker 1987; Grauel and Putz 2004; Pavlovic and Leicht-Young 2011; Addo-Fordjour and Rahmad 2015). Special attention was paid to the spread and invasion of lianas in many European countries quite recently. However, only three species of lianas, i.e. *Parthenocissus quinquefolia*, *Lonicera japonica* and *Vitis vinifera* have been included in the list of the 150 most widespread alien species in Europe (Lambdon et al. 2008), whereas at a country or regional level, the number of invasive and potentially invasive lianas is often higher. Fast spread and invasion of several species of the genus *Vitis* has been observed in Italy (Ardenghi et al. 2014; Ardenghi and Cauzzi 2015). *Pueraria montana* var. *lobata* (*P. lobata*) is considered to be invasive in Switzerland (Gigon et al. 2014). *Parthenocissus inserta* has been included in the Black List of the Czech Republic (Pergl et al. 2016).

Implementing the European Union Regulation 1143/2014 on invasive alien species, a horizon scanning for potentially invasive alien species has revealed 98 species that pose particular danger in the EU (Roy et al. 2015). *Celastrus orbiculatus* was listed among the alien species with a potentially negative impact on biodiversity and was prioritised for pest risk analysis (Roy et al. 2015; Tanner et al. 2017).

Celastrus orbiculatus was introduced to Europe as an ornamental plant at around 1860. Soon after its introduction, in 1863, it was first offered for sale to the public as an ornamental plant in Siebold's nursery (The Netherlands) catalogue (Del Tredici 2014). According to Adolphi et al. (2012), this species was first reported to have escaped in Germany in the early 1950s near Lohr am Main, but was then erroneously identified as *C. scandens*. To date, *C. orbiculatus* has been recorded as a casual or naturalised species in 13 European countries (Stace 1997; Verloove 2006; Purcel 2010; Pyšek et al. 2012; Tokarska-Guzik et al. 2012; Leonhartsberger 2013; Adolphi 2015; Beringen et al. 2017; Gudžinskas et al. 2017; Alberternst and Nawrath 2018, etc.).

Celastrus orbiculatus was introduced to the United States from Asia in the late 19th century, and has become naturalised in the Eastern States where it occupies large areas of disturbed temperate forests, alluvial woods, and roadsides (Merriam 2003; Pande et al. 2007; Leicht-Young et al. 2007, 2013; Kuhman et al. 2013; Horton and Francis 2014). This species causes changes in ecosystem productivity and structure (Ladwig and Meiners 2010; Hoosein and Robinson 2015), but most seriously it threatens the native congener, *C. scandens*, by hybridisation (Leicht and Silander 2006; Leicht-Young et al. 2007; Zaya et al. 2015). In New Zealand, *Celastrus orbiculatus* started to spread in 1981. Almost two decades later it became quite frequent and is considered as a threat to native ecosystems (Williams and Timmins 2003).

Quite recently a risk assessment of *C. orbiculatus* for the EU, with special focus on the Netherlands, has been performed (Beringen et al. 2017). However, this risk assessment was based mainly on studies from North America and on a few surveys on this species from several European countries. Important results of several other investigations that have not been evaluated in the previous risk assessment (Beringen et al. 2017) have revealed new important information about the invasiveness and performance of *C. orbiculatus* in the Baltic Region and in Central Europe (e.g. Purcel 2010, 2011; Gudžinskas et al. 2017; Alberternst and Nawrath 2018).

Despite the increased number of studies on *C. orbiculatus*, many of its biological and ecological properties, which have an important effect on the spread and emerging invasion, were insufficiently known in Europe. Therefore, we started to investigate established populations of *C. orbiculatus* in Lithuania, which are at the north-eastern limit of current naturalisation of this species in the southern part of the boreal biogeographic region of Europe. The aims of this study were (a) to analyse the current distribution of *C. orbiculatus* in Lithuania and Europe, (b) to evaluate the size of *C. orbiculatus* populations and occupied habitats in Lithuania, (c) to assess allocation of flowers by the gender; (d) to evaluate the age of individuals and approximate time of population establishment, (e) to study radial increment rates and possible relationships with meteorological conditions, (f) to investigate generative reproduction and sapling density in an invaded habitat. The results of this study provide information for a better understanding of *C. orbiculatus* reproduction, spread and invasion to natural, seminatural and human-made habitats.

Materials and methods

Study species

Celastrus orbiculatus Thunb. (Celastraceae R. Br.) is a deciduous woody vine that climbs by means of twining about a support. Cane-forming stems are located just above the ground and liana-forming stems are in the canopy layer where it climbs through the tree trunk and branches. (Leicht-Young et al. 2007; Zhixiang and Funston 2008). Flowers frequently are functionally unisexual because of abortion or reduction of male or female parts, thus the plants are usually dioecious, sometimes monoecious, though plants develop both unisexual and perfect flowers (Brizicky 1964; Gleason and Cronquist 1991; Burnham and Santana 2015). This species is native to East Asia and its range includes Central and North Japan, Korean Peninsula, Far East of Russia and China north of the Yangtze River and tolerates a very wide range of climates (Ohwi 1965; Williams and Timmins 2003; Zhixiang and Funston 2008).

Mapping of distribution

A distribution map of *C. orbiculatus* in Lithuania was compiled applying a system of grid cells, which were arranged according to geographical coordinates with sides of 6' of latitude and 10' of longitude (Gudžinskas 1993). A distribution map of *C. orbiculatus* in Europe was compiled using data of available published reports (hereafter referred to in the text). We also used data from our studies and some unpublished (acknowledged personal reports, herbaria) information on occurrences of this species. The map was created employing the same base-map, grid cells and "AFE Editor" software as used for the "Atlas Florae Europaeae" map compilation (Kurtto et al. 2004).

Study sites

We performed field studies on four populations of *C. orbiculatus* in Lithuania during the growth season of 2016 and flower gender was studied in June of 2018. We also surveyed a newly discovered population in Vandžiai (Raseiniai distr.) in September of 2018. Therefore, population size and habitats of *C. orbiculatus* were evaluated in five sites in Lithuania (Table 1). Additionally, in July 2019, we surveyed two previously unregistered sites of *C. orbiculatus* in Girionys (Kaunas distr., Lithuania) and in Ķemeri village (Jūrmala city, Latvia). Herbarium specimens of this species from all sites were collected by the authors and deposited at the Herbarium of the Institute of Botany of the Nature Research Centre (BILAS).

Sampling procedures

Population size and habitat characteristics

The areas of small populations were measured using measurement tape, whereas the size of large populations with complicated configuration was measured by applying geographical coordinates established at certain points of the stand perimeter. The area occupied by *C. orbiculatus* was calculated using online software provided from the Spatial Information Portal of Lithuania (www.geoportal.lt). The quantity of fruits was estimated visually. When solitary fruits were found on the lateral branches of a *C. orbiculatus* individual, its fruit yield was classified as poor, whereas for individuals having one or more fruits on most of their axillary cymes on a lateral branch, its fruit yield was classified as abundant.

The height of trees in the habitats and height of *C. orbiculatus* in the trees were measured with a height measurement device (Haglöf EC II, Sweden). Coverage of plants (in per cent) of different vegetation layers was estimated visually. We identified habitat types following the Interpretation Manual of European Union Habitats (Rašomavičius 2012; European Commission 2013) according to the ecological features, vegetation structure and species composition.

Table 1. Geographical characteristics of the studied *Celastrus orbiculatus* sites in Lithuania.

Site	Latitude (N) / Longitude (E)	Altitude (m a.s.l.)
Paneriai (Vilnius city)	54.6344, 25.1526	150
Visoriai (Vilnius city)	54.7403, 25.2606	174
Babrungėnai (Plungė distr.)	55.9986, 21.8699	158
Palanga city	55.9591, 21.0915	10
Vandžiai (Raseiniai distr.)	55.3017, 23.4276	58

Analysis of flower gender

Flower gender of *Celastrus orbiculatus* was studied in four populations (Babrungėnai, Palanga, Paneriai and Visoriai) at the beginning of June of 2018. We sampled from two to five mature and clearly identifiable separate individuals from each population, 12 individuals in total. Ten lateral branches (usually 15–20 cm long) at 1–3 m high from the ground with several inflorescences were taken from each plant, labelled and placed into separate plastic bags and brought to the laboratory for further analysis. At the laboratory, the number of inflorescences and number of flowers in each inflorescence was counted starting from the base of branch and inflorescence. Each flower was studied under the binocular microscope and dissected, when necessary, to estimate its gender. Flowers with developed anthers and undeveloped pistil and ovary were treated as functionally male, flowers with developed pistil and ovary but undeveloped anthers were treated as functionally female flowers (Figure 1).

Study of seedlings and saplings

The density of *C. orbiculatus* seedlings and saplings was estimated in three parallel 20-meter-long transects in Paneriai Forest in early September 2016. The transects were at a distance of approx. 30 m between the previous transect. Transects were selected in an area free of ground cover of *C. orbiculatus* shoots. The first transect was arranged in the central part of the *C. orbiculatus* stand with the densest and highest mature individuals, the second transect was selected at the edge of the stand with mature individuals, and the third transect was in an area free of mature *C. orbiculatus* individuals. The transects were divided into 20 sampling plots of 1 m^2 using a frame with all sides of 1 m. The number of seedlings and saplings grown from seeds was counted. Individuals grown from seeds in the year of the study were ascribed to the group of seedlings, whereas elder individuals (two or more years old) were identified as saplings. We distinguished seedlings from saplings by the level of stem lignification. Saplings and individuals grown from underground shoots were distinguished by the shape of their leaves and the character of the shoot. In each transect, we also measured the height of 10 randomly selected seedlings and 10 saplings.

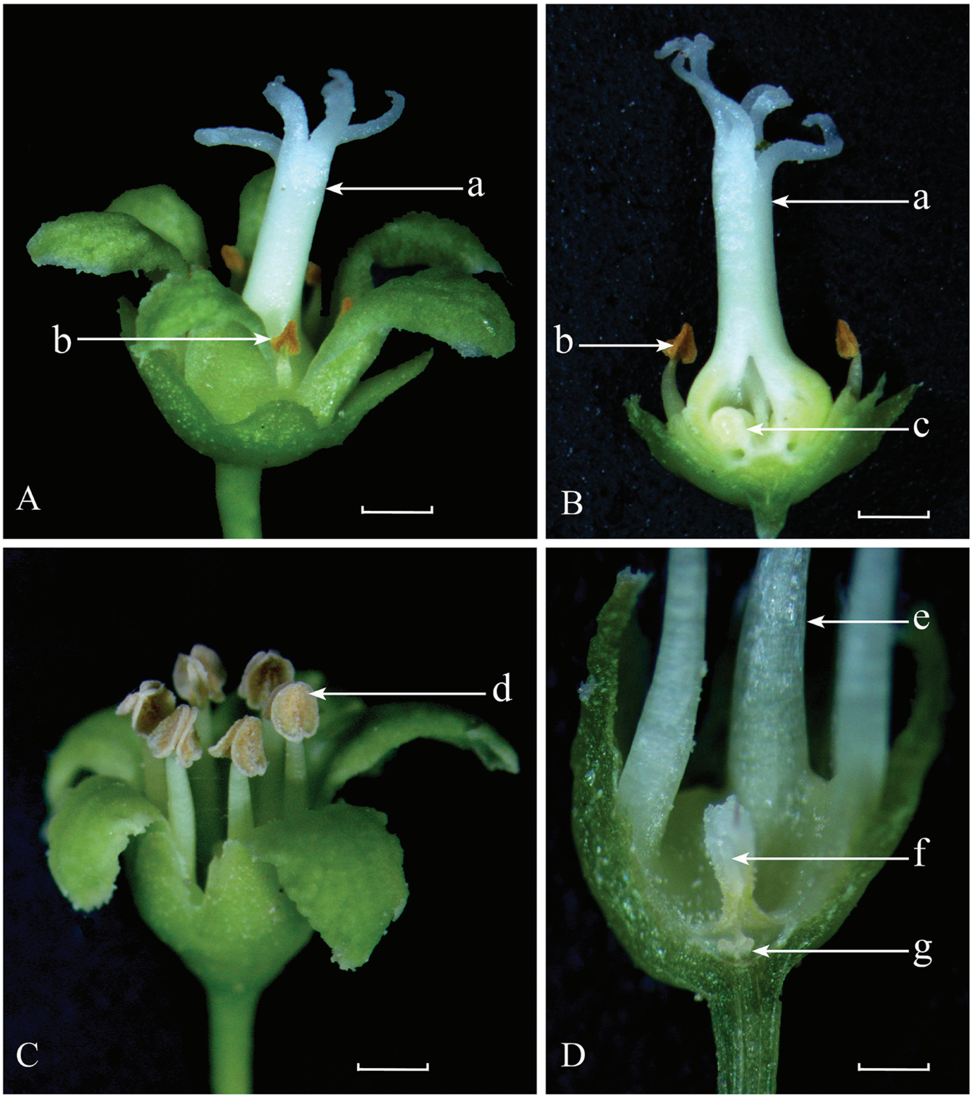

Figure 1. Flowers of *Celastrus orbiculatus*. **A** Functionally female flower with developed pistil (**a**) and staminodes (**b**) **B** longitudinal section of functionally female flower (petals removed) with developed pistil (**a**), staminodes (**b**) and ovules in the ovary (**c**) **C** functionally male flower with developed stamens (**d**) **D** longitudinal section of functionally male flower with filaments of developed stamens (**e**), pistillode (undeveloped pistil, **f**) and ovary with aborted ovules (**g**). Scale bars: 1 mm (**A–C**); 2 mm (**D**).

Evaluation of plant age and radial growth

Stems of *C. orbiculatus* for dendrochronological analysis were sampled in three populations (Babrungėnai, Visoriai and Paneriai) at the end of the growth period in September 2016. The population of Palanga was not sampled the same way because of an insufficient number of primary stems. Two series of samples were collected in the population of Paneriai Forest: the first series was collected in the - presumably - oldest part of the population, another series in the peripheral part of the stand, at 40–50 m from the first sampling site. Ten of the largest individuals from each sampling area were selected and 15–20-cm-long stem sections were cut with a saw at ground level. Samples were numbered, diameters measured with a caliper and placed into paper bags for drying at ambient temperature for three months.

Before counting annual rings, sections of ca. 5 cm were cut from each dried sample of the stem and one of its surfaces sanded and polished. Polished surfaces of the samples were stained with original stain prepared from the powder of *Curcuma* rhizomes boiled in vegetable oil (5 g of powder and 20 ml of vegetable oil). Prepared cross-sections of *Celastrus orbiculatus* stems were examined under a binocular microscope (LEICA EZ4), the annual rings of the xylem were counted and the width of annual rings was measured with an accuracy of 0.1 mm.

Information on the monthly minimum temperatures and the amount of precipitation in the period from 1989 to 2016 was provided by the Hydrometeorological Service under the Ministry of Environment of Lithuania. The data was used to evaluate the impact of meteorological factors on the radial stem growth of *C. orbiculatus*.

Statistical analyses

The normality of data distribution was evaluated using the Shapiro-Wilk test. Comparison of normally distributed data sets (pooled seedling and sapling density, width of annual rings) was performed applying ANOVA several-sample F-test and Tukey's post-hoc pairwise comparisons. Two normally distributed data sets of seedling and sapling height were compared applying Student's t-test. Because seedling and sapling density in the studied plots were distributed non-normally, comparison of their densities was analysed applying non-parametric Kruskal-Wallis H-test and Mann-Whitney post-hoc pairwise comparisons. Correlations between normally distributed data sets of annual ring width and meteorological parameters were calculated applying Pearson's rank correlation test. The significance level of statistical tests was set at $p < 0.05$. Dependence of stem age and its diameter were evaluated using linear bivariate model. Descriptive statistical analysis results include mean values and standard deviations (mean ± SD), in analyses of seedling and sapling density also including minimum, maximum and median. All calculations were performed using PAST 3.20 (Hammer et al. 2001).

Results

Distribution

Currently, *C. orbiculatus* occurs in six localities in southeastern (Paneriai and Visoriai, Vilnius city), central (Vandžiai, Raseiniai distr. and Girionys, Kaunas distr.) and western (Babrungėnai, Plungė distr. and environs of Palanga city) parts of Lithuania

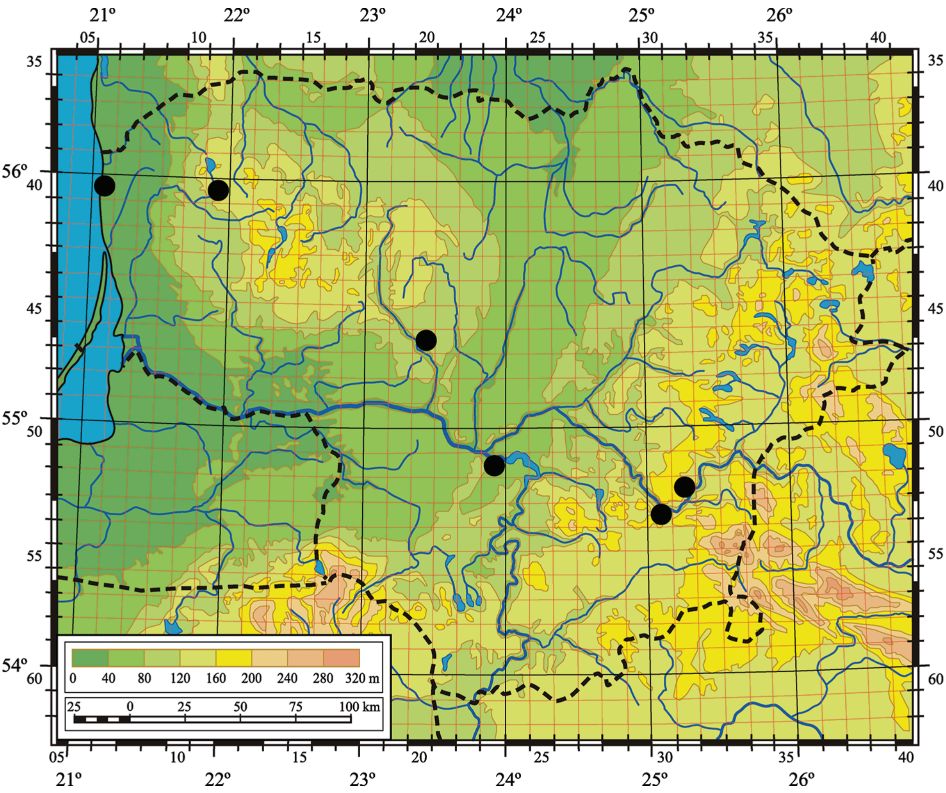

Figure 2. Current distribution of *Celastrus orbiculatus* in Lithuania mapped on to the local grid.

(Figure 2). This species was first recorded in the environs of Palanga city in 2014. In the same year, *C. orbiculatus* was also found in Visoriai. Two localities of this species were recorded in 2016 (Babrungėnai and Paneriai). Localities in Vandžiai and Girionys were discovered in 2018 and 2019, respectively.

Information on the distribution of *C. orbiculatus* in Europe was quite dispersed over numerous publications and other sources of information. According to these sources *C. orbiculatus* has been recorded in 13 European countries: Austria (Leonhartsberger 2013; Sauberer and Till 2015), Belgium (Verloove 2006, 2013), the Czech Republic (Červinka and Sádlo 2000; Pyšek et al. 2012; Pergl et al. 2016), Germany (Brandes 2011; Adolphi et al. 2012; Adolphi 2015; Alberternst 2018; Alberternst and Nawrath 2018), Latvia, Lithuania (Gudžinskas et al. 2017), the Netherlands (Beringen et al. 2017), Norway (Gederaas et al. 2012; Beringen et al. 2017), Poland (Purcel 2010, 2011), the European part of Russia (Morozova 2014, without exact locality), Sweden (Beringen et al. 2017), Ukraine and United Kingdom (Stace 1997; Beringen et al. 2017; BSBI 2018). In total, 58 sites of occurrence of this species have been reported so far and they fall into 42 grid cells of the "Atlas Florae Europaeae" (Figure 3).

Analysis of the spread dynamics of registered *C. orbiculatus* populations in Europe revealed very slow increase of sites of occurrence (on average three new sites per 10 years)

Figure 3. Distribution of *Celastrus orbiculatus* in Europe mapped on to the grid of the *Atlas Florae Europaeae*.

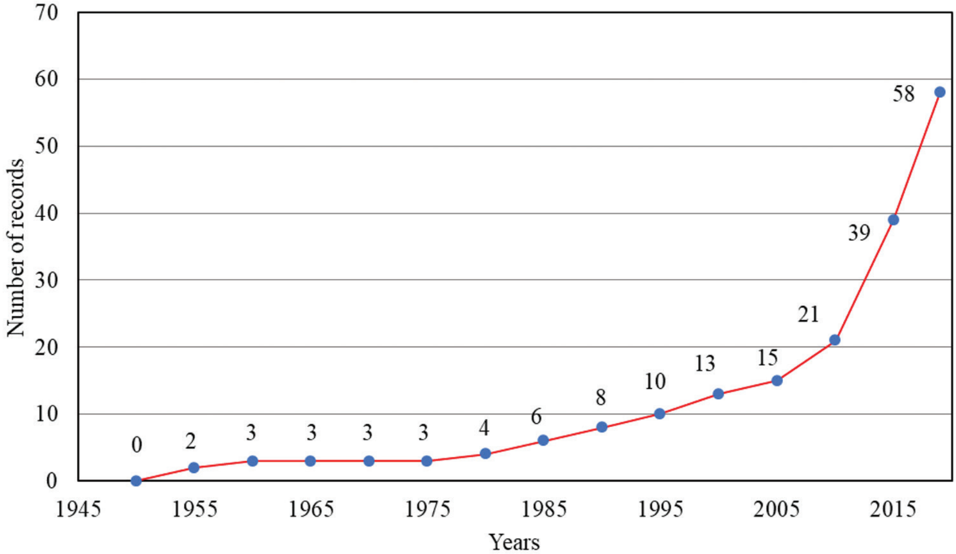

Figure 4. Cumulative curve of *Celastrus orbiculatus* record number in Europe.

from its first report in Europe in 1953 (Adolphi et al. 2012) until 2005; this period corresponds to the lag phase. From 2005 onwards, the number of registered new occurrences started to increase rapidly, on average almost three sites per year (Figure 4). The increase of occurrences since 2005 may be recognised as the start of the exponential growth phase.

Habitats

Four of the studied populations (Paneriai, Visoriai, Babrungėnai and Vandžiai) were in forest habitats and one population (Palanga) on the edge of a forest. In Paneriai Forest, the plants occupied an approximately 70-year-old *Pinus sylvestris* stand with admixture of *Acer platanoides* and *Quercus robur* in the second tree layer (Table 2). The population in Visoriai was recorded in an approximately 60-year-old *Betula pendula* stand with admixture of *Picea abies* and *Populus tremula* in both tree layers. In Babrungėnai, the population of *Celastrus orbiculatus* occupied a forest glade under a low-voltage power transmission line and a surrounding approximately 50-year-old tree-stand of almost equal proportions of *Pinus sylvestris*, *Picea abies* and *Betula pendula*. The stand of *C. orbiculatus* in Vandžiai (Raseiniai distr.) occupies an about 20-year-old woodland composed of *Betula pendula*, *Alnus incana* and *Tilia cordata*. The stand was in a transitional zone between broad-leaved forest and alluvial forest habitats (Table 2). Near Palanga, the stand of *Celastrus orbiculatus* was on a shrubby edge of the dry pine forest on dunes and occupies a transitional area between the forest and sandy grassland.

The studied forest habitats invaded by *C. orbiculatus* had characteristic vertical structure of the tree and shrub layers and their cover (excluding *C. orbiculatus*) ranged from 40% to 60% and from 30% to 60%, respectively (Table 2). Normal development of the tree and particularly of the shrub layer suggests that this species invaded already stabilised habitats without significant disturbances.

In Lithuania, *C. orbiculatus* occurs in quite different types of habitats. The population in the environs of Palanga occupies a transitional area between a habitat of wooded dunes (2180 Wooded dunes of the Atlantic, Continental and Boreal region) and sand grasslands (6120* Xeric sand calcareous grasslands). In Paneriai, this species occupies mature pine forest (9010* Western Taiga) and the transitional zone to spruce forest (9050 Fennoscandian herb-rich forests with *Picea abies*). In Visoriai, *C. orbiculatus* grows in stadial forest dominated by *Betula pendula* with an admixture of *Picea abies*, which according to the ecological conditions and species composition is close to the herb-rich spruce forest habitat (9050 Fennoscandian herb-rich forests with *Picea abies*). The stand of *C. orbiculatus* in Vandžiai invaded a young *Betula pendula* stand situated in the transitional zone between alluvial forest (91E0 Alluvial forests with *Alnus glutinosa* and *Fraxinus excelsior*) and broad-leaved forest (9020* Fennoscandian hemiboreal natural old broad-leaved deciduous forests).

Population size and characteristics

In Paneriai Forest, a dense stand of this species with mature individuals occupies an area of 2600 m^2. The total area of the stand, including recorded seedlings and saplings, comprises 3640 m^2. At the Vandžiai site (Raseiniai distr., Central Lithuania) the species occupies 880 m^2 while the other studied stands of *C. orbiculatus* were significantly smaller (Table 2). Thus, the total area occupied by this species in all registered populations in Lithuania approximates to 0.51 ha.

Table 2. Characteristics of the studied *Celastrus orbiculatus* sites.

Character	Site				
	Paneriai	Visoriai	Babrungėnai	Palanga	Vandžiai
Area occupied by the stand of *Celastrus orbiculatus* (m^2)	3640	480	90	20	880
Age of the dominant trees (years)	70	60	50	–	20
Maximum height of the tree layer (m)	24	17	15	–	15
Coverage of the tree layer (%)	60	50	50	–	40
Coverage of the shrub layer (%)	60	30	60	20	50
Coverage of the herb layer (%)	40	30	30	60	20
Coverage of the bryophyte layer (%)	60	10	5	20	5
Maximum height of *Celastrus orbiculatus* (m)	18	14	10	3	14
Coverage of *Celastrus orbiculatus* in the tree layer (%)	15	20	10	–	20
Coverage of *Celastrus orbiculatus* in the shrub layer (%)	50	40	30	10	60
Coverage of *Celastrus orbiculatus* in the herb layer (%)	60	30	60	50	60
Total coverage of *Celastrus orbiculatus* in all layers (%)	70	60	70	50	70

In all studied sites, *C. orbiculatus* shoots were distributed over all vegetation layers and in all cases it was the dominant species in the entire community. Its total coverage ranged from 50% to 70% (Table 2). In four studied sites, shoots of *Celastrus orbiculatus* reached the tree canopy layer and the height of the tallest individuals ranged from 10 to 18 m, though its coverage in this layer was quite low (Table 2). Much higher coverage of *C. orbiculatus* in forest habitats was in the shrub layer, ranging from 30% to 60% (mean 45%). The highest mean coverage (52%) of this species was recorded in the herb layer, where it ranged from 30% to 60%.

Flower gender

Analysis of flower (n = 1913) gender of 12 individuals revealed that all plants were monoecious, whereas dioecious or polygamo-dioecious individuals were not found. In all studied individuals either functionally female or male flowers prevailed, from 74.2% to 84.3% and from 76.5% to 84.9%, respectively (Table 3). Individuals with mostly functionally female or male flowers were recorded in the Paneriai and Palanga populations, whereas the Visoriai and Babrungėnai populations consisted of only individuals with prevailing functionally male flowers.

We noted that functionally female flowers of individuals with prevailing male flowers were usually arranged in the proximal part of lateral branches, at the first to the third node, and in the proximal part of the inflorescence. In individuals with prevailing female flowers, functionally male flowers were usually arranged in inflorescences at the distal part of a branch or at the apex of the inflorescence.

The existence of monoecious individuals in all studied sites explains the pattern of *C. orbiculatus* fructification. At the Babrungėnai and Visoriai sites, the set of *C. orbiculatus* fruits in 2016 was poor because dioecious individuals bearing only dominant male flowers were recorded. In Palanga and in Paneriai, some individuals produced

Table 3. Distribution of flowers by gender in the studied individuals of *Celastrus orbiculatus* from four populations in 2018.

Site	Number of individuals	Number of studied flowers	Prevailing gender of flowers	Gender of flowers			
				female		male	
				number	%	number	%
Paneriai	1	138	male	25	18.1	113	81.9
	2	147	female	116	78.9	31	21.1
	3	124	female	92	74.2	32	25.8
	4	161	female	135	83.9	26	16.1
	5	235	female	198	84.3	37	15.7
Visoriai	1	156	male	27	17.3	129	82.7
	2	131	male	23	17.6	108	82.4
	3	166	male	39	23.5	127	76.5
Babrungėnai	1	152	male	30	19.7	122	80.3
	2	126	male	19	15.1	107	84.9
Palanga	1	170	male	32	18.8	138	81.2
	2	207	female	171	82.6	26	17.4

abundant fruits in 2016. In both these sites individuals with dominant functionally female and dominant functionally male flowers were registered.

Seedlings and saplings

In all sampling plots of the central part of the *C. orbiculatus* stand, we recorded 162 seedlings and saplings, and their mean density was 8.10 ± 1.94 individuals/m^2 (Table 4). ANOVA test revealed significant differences among the studied transects ($F(2, 57) = 81.29$, $p < 0.001$). In the periphery of the stand, where generative individuals were absent, and in transects located outside the stand, the density of seedlings and saplings was statistically significantly lower than in the central part of the stand (Tukey's pairwise comparison, $Q = 13.81$; $p < 0.001$ and $Q = 16.95$, $p < 0.001$, respectively). The difference between density of seedlings and saplings at the periphery of the stand and outside of the main stand was statistically insignificant ($Q = 3.14$, $p = 0.076$). Nevertheless, in both these transects seedling and sapling density was considerable so that one can expect further extension of the population (Table 4).

Analysis of the proportions of seedlings and saplings revealed that in the central part of the stand saplings prevailed (55.55%), whereas on the periphery and outside the main stand seedlings prevailed (54.10% and 52.63%, respectively). Density of seedlings and saplings in the centre of the stand was statistically significantly higher than in the other two transects (Table 5). However, we did not find significant differences of seedlings and saplings between transects on the periphery and outside the stand (Mann-Whitney U-test, $U = 138.00$, $n = 40$, $p = 0.081$).

Two- and three-year-old saplings were almost equally presented in all transects. Four-year-old saplings were recorded in the central part of the stand only. The height of the

Table 4. Total number of *Celastrus orbiculatus* seedlings and saplings in the studied transects (Paneriai Forest, Vilnius), their density (mean ± SD), minimum and maximum number in sampling plot and median. The same letter indicates statistically significant differences according to Tukey's pairwise comparison ($p < 0.001$).

Transect location	Sampling plots (n)	Total number	Density (individuals/m^2)	Minimum	Maximum	Median
Centre	20	162	8.10 ± 1.94^{ab}	5	12	8
Periphery	20	61	3.05 ± 1.50^{a}	0	6	3
Outside	20	38	1.90 ± 1.41^{b}	0	5	2

Table 5. Number, percentage and density (mean ± SD, individuals/m^2) of *Celastrus orbiculatus* seedlings and saplings in three transects in Paneriai Forest (Vilnius). The same letter indicates statistically significant differences according to Mann-Whitney pairwise comparison ($p < 0.001$).

Transect location	Seedlings			Saplings		
	Number	%	Density	Number	%	Density
Centre	72	44.45	3.60 ± 1.67^{ab}	90	55.55	4.50 ± 1.15^{ab}
Periphery	33	54.10	1.65 ± 1.26^{a}	28	45.90	1.4 ± 0.88^{a}
Outside	20	52.63	1.00 ± 0.97^{b}	18	47.37	0.90 ± 0.91^{b}

measured seedlings ranged from 4 cm to 10 cm. Mean height of seedlings was 6.70 ± 1.58 cm. The height of saplings ranged from 9 cm to 19 cm and their mean height was 13.03 ± 2.70 cm. Saplings were significantly taller than seedlings (Student's t-test, $t = 11.07$, $n = 60$, $p < 0.001$). Two four-year-old saplings were 18 cm and 19 cm in height.

Age of stands and individuals

According to the number of annual rings, the oldest analysed population of *C. orbiculatus* was that at Visoriai, in which the oldest recorded living stem was 21 years old; the oldest dead stem was 30 years old, and mean age of sampled stems was 11.9 ± 4.7 years (Table 6). Thus, the first individual in this population settled here in 1987. The second oldest population was that at Paneriai and the oldest sampled stem in the central part of the stand was 18 years old. Therefore, the population of Paneriai Forest established itself around 1999. The age of sampled stems in the central part of the site ranged from 11 to 18 years. The age of the largest sampled stems in the periphery of the stand ranged from 6 to 11 years (Table 6). The population of *C. orbiculatus* at Babrungėnai was younger than at the other sites and the oldest stem was 11 years old. Thus, this population originated there as late as 2006. As the population at Palanga was quite small, we sampled one of the largest stems that was 10 years old. Thus, this population also originated in 2006.

Table 6. Age and stem diameter of studied *Celastrus orbiculatus* stems (n = 10 in each stand) and estimated year of the stand initiation.

Features	Visoriai	Paneriai		Babrungėnai
		Centre	Periphery	
Age of the oldest living stem (years)	21	18	11	11
Age of the youngest stem (years)	7	11	6	3
Age of the oldest dead stem (years)	30	–	–	–
Estimated year of the stand initiation	1987	1999	2006	2006
Diameter of the oldest living stem (mm)	47.5	42.1	18.0	15.7
Mean age of stems (years)	11.9 ± 4.7	13.2 ± 2.3	9.6 ± 1.9	6.9 ± 3.0
Mean diameter of stems (mm)	20.4 ± 12.8	25.8 ± 8.7	14.6 ± 2.7	11.99 ± 2.4

Stem diameter and radial increment

The diameter of sampled stems of *C. orbiculatus* was strongly correlated with their age (R^2 = 0.73, $p < 0.001$, n = 40). However, analysis of same age stem diameters revealed a significant variation. The diameter of 11-year-old stems (n = 10), comprising the largest age group among the sampled stems, ranged from 11.2 mm to 25.4 mm (mean 17.7 ± 3.9 mm). Variation of diameter of same aged stems in the same habitat suggests a significant effect of the stem position and competition with other stems, height of foliage, light availability, etc.

The mean width of annual rings (n = 376) of the xylem of *C. orbiculatus* of all studied stems (n = 40) was 0.77 ± 0.26 mm. The minimum width of annual rings in the studied plants was 0.3 mm, the maximum width was 1.7 mm, and the median width of annual rings was 0.7 mm. We compared the width of annual rings formed from 2006–2016 and found no statistically significant differences among years (F (10, 99) = 0.56, p = 0.84). Also, differences could not be detected by pairwise comparison between years. However, we found statistically significant differences among annual ring width of the same age (11-year-old) stems (F (9, 100) = 16.93, $p < 0.001$). Pairwise comparison also revealed significant differences between annual ring width in stems both from the same site and from different sites.

Results from the analysis of the relationships of annual ring width and radial increment of the stem diameter with meteorological factors were quite unexpected. No statistically reliable correlations were found between annual ring widths and mean annual temperature (r = 0.16, p = 0.64), mean winter temperature (r = –0.01, p = 0.84), mean summer temperature (r = 0.21, p = 0.53), minimum winter temperature (r = –0.10; p = 0.43), and annual precipitation (r = 0.12; p = 0.72).

Discussion

Distribution

The currently recorded localities of *C. orbiculatus* in Lithuania are in different regions of the country and separated by distances ranging from 10 km to 130 km. Therefore, we suppose that escaped populations of *C. orbiculatus* have originated from different sources. Considering the common cultivation of this species in gardens and for landscaping in Lithuania (Navasaitis 2008), Latvia (Laiviņš et al. 2009) and other European countries (Stace 1997; Beringen et al. 2017), its further spread is expected. The species is dispersed by several vectors, including birds (Purcel 2011; Alberternst 2018; Alberternst and Nawrath 2018); thus, we can presume that this species is more widely distributed, but that many existing populations still have not been identified.

Beringen et al. (2017) questioned whether new sites of *C. orbiculatus* in Europe originate from the dispersal of seeds by berry-eating birds, or from the dumping of garden waste. Studies by Purcel (2011), Alberternst (2018), and Alberternst and Nawrath (2018) confirmed that birds are important vectors for spreading this species both in proximity of the invaded area as well as at quite long distances. We suppose that at least one of the Lithuanian sites in Babrungėnai, situated under low-voltage power lines, is a result of bird dispersal from areas of cultivation. However, we were not able to identify a source of seeds in the area around Babrungėnai (within 1 km range). There is also a high probability that birds have dispersed seeds of *C. orbiculatus* from Vilnius Botanical Garden or another place of cultivation to Paneriai Forest (Vilnius). The nearest known place of cultivation is about 8 km away. In Visoriai, *C. orbiculatus* seeds or vegetative parts could have been introduced via wastes from gardens that are located about 0.5 km away from the site. In the environs of Palanga, *C. orbiculatus* was probably introduced from a place of cultivation which is approx. 0.8 km to the north. Seeds or fruits could have been brought in by strong winds, birds or human activity. The population at Vandžiai is probably a relic of former cultivation.

The map of its current distribution (Figure 3) shows higher concentrations of occurrences in western and eastern Germany (17 sites of the total 58 sites registered till the end of 2019), southern Sweden, western Poland and Lithuania. Our analysis of the occurrence and distribution of this species in Central Europe leads us to consider that this plant should be classified as having a restricted range. If the rate of spread and records of new occurrences continue at the same rate as during the last 15 years, *Celastrus orbiculatus* may become widespread in Europe within the next decade.

Habitats

In its native range in East Asia, *C. orbiculatus* grows mainly in mixed forests, along forest margins and on grassy slopes (Zhixiang and Funston 2008). In the invasive range in North America, it occupies a very wide range of habitats: wet and dry forests, old-growth forests, areas of forests damaged by wind-throws, abandoned fields, reforested areas, slopes, dunes and various anthropogenic or heavily disturbed habitats (Leicht-Young et al. 2007; Pavlovic and Leicht-Young 2011, etc.). Similar habitats are occupied in New Zealand (Williams and Timmins 2003). Studies of *C. orbiculatus* spread in North America have revealed that coniferous forests are less favourable habitats and large areas of these forests may act as a barrier against spread of this species (Merow et al. 2011).

However, results of our study suggest that forest habitats dominated by coniferous trees are suitable for *C. orbiculatus* and forests belonging to the western taiga, wooded dune and herb-rich spruce forest habitats are also prone to the invasion of this liana.

In Europe, *C. orbiculatus* most frequently has been found in areas close to urban environments and occupying human-made or disturbed habitats; however, in Austria, Germany and Poland this species has been found occurring in natural or seminatural habitats located at significant distances from urban areas (Purcel 2010; Adolphi et al. 2012; Leonhartsberger 2013; Adolphi 2015; Sauberer and Till 2015; Alberternst and Nawrath 2018, etc.). Beringen et al. (2017) concluded that *Celastrus orbiculatus* is able to establish itself and invade alluvial (91E0 Alluvial forests with *Alnus glutinosa* and *Fraxinus excelsior*) and riparian (91F0 Riparian mixed forests of *Quercus robur*, *Ulmus laevis* and *Ulmus minor*, *Fraxinus excelsior* or *Fraxinus angustifolia*, along major rivers) forest habitats on moist soils. In Lithuania, *C. orbiculatus* was found to invade a wider range of natural habitats than has been reported from other European countries. The ability of this species to invade dry dune woodlands, grasslands, pine, spruce alluvial and riparian forests of high conservation value is of particular concern. Due to the ability of this species to establish itself in alluvial and riparian habitats, it may very well intensively spread along watercourses in the future.

Celastrus orbiculatus affects all layers of the vegetation in the invaded habitats. Its coverage in the tree canopy layer was quite low in Lithuania, although it substantially reduces light availability in the lower vegetation layers. We detected the highest coverage of *Celastrus orbiculatus* in the shrub and herb layers. Even higher total coverage, ranging from 80% to 100%, of this species has been recorded in Hessen, Germany (Alberternst and Nawrath 2018). This species hinders light penetration and interferes with all plant growth, especially of shrubs and young trees. *Celastrus orbiculatus* also affects mature and young trees and shrubs by girdling their stems and hindering organic matter flow in the phloem. Furthermore, *C. orbiculatus* increases the load of biomass on the upper part of a tree, thus increasing the likelihood of wind damage (Horton and Francis 2014).

Population size and characteristics

Registered populations of *C. orbiculatus* currently occupy an area of 0.51 ha in Lithuania. However, information on the area occupied by this species in other European countries is incomplete. Probably the largest area occupied by *C. orbiculatus* is in Poland, Lubuskie province. There, about 170 ha in the environs of World War II fortifications are occupied by *C. orbiculatus* with very dense stands on about 9 ha (Purcel 2010). In Neuwied (Germany), a stand of *C. orbiculatus* occupies about 2 ha along a railway (Adolphi et al. 2012). Alberternst and Nawrath (2018) estimated that in Hessen (Germany) stands of various density of *C. orbiculatus* occupied ca. 44.6 ha in 2017. In Austria, in the environs of Graz, a stand of *C. orbiculatus* stretches over a length of 50 m along the River Mur and there the liana climbs in trees and covers riparian areas (Leonhartsberger 2013). The total area occupied by *C. orbiculatus* in all European countries is currently estimated at approx. 250 ha. However, as new occurrences of *C. orbiculatus* are being recorded in Europe almost every year, the actual distribution and occupied area could very well be larger than currently known.

Flower gender

Individuals of *C. orbiculatus* are known to be either functionally dioecious (Brizicky 1964), functionally monoecious (Hou 1955) or polygamo-dioecious (Gleason and Cronquist 1991). Our study, though based on only 12 sampled mature individuals, revealed that only monoecious individuals were present in the four studied populations in Lithuania. The fact that in all studied populations we found monoecious individuals explains the presence of a certain amount of ripe fruits (Gudžinskas et al. 2017). Interestingly, in 2019 we recorded an individual of *C. orbiculatus* in Ķemeri (Latvia) with solitary fruits and we suppose that this plant is also monoecious with prevailing functionally male flowers.

The available information on the generative reproduction of *C. orbiculatus* in Europe is controversial. Verloove (2013) noted that *C. orbiculatus* hardly flowers in Belgium and, therefore, its further spreading by sexual reproduction would seem rather unlikely. But, in contrast, at least in some of the sites in Germany, *C. orbiculatus* flowers and produces quite large amounts of seeds (Adolphi et al. 2012; Adolphi 2015). Purcel (2010, 2011) recorded generative spread of this species within and around large stands as well as in a range of 3–5 km from the sites of initial introduction. Abundant reproduction by seeds has been recorded in Hessen, Germany (Alberternst and Nawrath 2018). The different reproductive behaviour of *C. orbiculatus* reported from various regions of Europe may possibly be caused by the different structure of populations according to the gender of flowers (Hou 1955; Brizicky 1964; Gleason and Cronquist 1991; Burnham and Santana 2015). Regional differences of the gender allocation in *C. orbiculatus* populations can be a result of separate introduction events from different sources.

The existence of monoecious individuals of *C. orbiculatus* in Lithuania supports the assumption that monoecious or polygamo-dioecious plants in other populations in Europe also exist. Thus, when monoecious or polygamo-dioecious individuals occur in an area, there are no obstacles for their sexual reproduction (Herron et al. 2007; Ladwig and Meiners 2010; Burnham and Santana 2015), further spread and invasion even in cases of solitarily growing individuals.

Seedlings and saplings

Celastrus orbiculatus does not form a persistent seedbank; almost all seeds germinate in spring and the light intensity does not affect their germination (Dreyer et al. 1987; Van Clef and Stiles 2001; Ellsworth et al. 2004a; Beringen et al. 2017). Relatively high proportions of seedlings survive under poor illumination conditions and seeds can wait for a certain time for forest habitat disturbances and creation of favourable light conditions for their proliferation (Greenberg et al. 2001; Ellsworth et al. 2004b).

Our research revealed significant densities of *C. orbiculatus* seedlings and saplings within limits of its dense stand with mature individuals, on the periphery and in proximity of the stand without mature individuals. The quite slow growth rate during the first three to four years, as revealed by our study, confirms that saplings form so-called seedling banks (Ellsworth et al. 2004b). We were not able to establish whether elder saplings were absent because of their death, poor seed production in certain years and absence of generative reproduction, or because on the fifth year they produce long shoots and become undistinguishable from shoots grown from roots and underground stems. Nevertheless, the results of this study revealed that *C. orbiculatus* is capable of reproducing by seeds and thus may easily expand its population even in undisturbed forest habitats.

There is insufficient information regarding the time point when individuals of *C. orbiculatus* grown from seeds reach maturity. Silveri et al. (2001) reported that in North America in newly established populations of seed origin, individuals of 12 years of age reached the generative stage. Seedlings and saplings grow slowly in the first few years, at least in forest habitats. Our study revealed that four-year-old saplings in a dense stand were up to 19 cm high. Under favourable light conditions saplings grow faster than under the canopy (Silveri et al. 2001). In the population of Palanga, a single sampled individual in a well-illuminated habitat on the forest edge was eight years old and produced fruits. Therefore, we conclude that individuals grown from seeds in well-illuminated habitats can reach the reproductive stage at approx. 10 years. Individuals grown from root-suckers grow very fast and four-year-old shoots can already reach the reproductive stage (Silveri et al. 2001; Ellsworth et al. 2004a).

Propagule pressure is among the most significant factors affecting the spread and invasiveness of plant species (Warren et al. 2013). Extensive cultivation as well as increase of naturalised populations of *C. orbiculatus* in Europe plausibly increase its propagule pressure on the native habitats and threatens further invasion.

Age of stands and individuals

The dendrochronological methods employed in this study enabled us to determine the approximate time of establishment of populations of *C. orbiculatus* and the age of their generative maturity.

Studies on the age structure of *C. orbiculatus* in the area of the Hudson River Estuary (New York State, USA) revealed that the oldest individuals were about 20 years old and their diameter was up to 70 mm, while the mean stem diameter of 5–7-year-old individuals was 17.5 mm (Hoosein and Robinson 2015). In New Zealand, the main stems of *C. orbiculatus* were commonly 50–60 mm in diameter, and the largest recorded stem was 140 mm in diameter. Most of the studied stems in New Zealand were 10–12 years old, whereas one stem collected on North Island at Clova Bay had 32 visible annual rings (Williams and Timmins 2003).

We found that the oldest living stem in the studied populations in Lithuania was 21 years old, and a dead stem (most probably quite recently strangled by other stems of *C. orbiculatus*) was 30 years old. The diameter of the oldest living stem was 47.5 mm. Significant differences in stem diameters in the area of the Hudson River Estuary, USA (Hoosein and Robinson 2015), on the North Island of New Zealand (Williams and Timmins 2003), and plants studied in Lithuania may be due to different habitat conditions. The individuals that we studied grew in undisturbed forest habitats, whereas in the USA and New Zealand most of the sampled plants grew in variously disturbed or forest fringe habitats (Williams and Timmins 2003; Hoosein and Robinson 2015). Thus, slower growth rate and, therefore, stem diameter, in Lithuania was probably caused not only by significant differences in climatic conditions as compared to those in the area of the Hudson River Estuary and in New Zealand, but also by stronger competition of the native species and light availability. Unfortunately, there are no published data on the age, stem diameter, and radial increment of *C. orbiculatus* from other regions of Europe and habitats within the plant's native range.

Stem diameter and radial increment

We analysed the effect of the meteorological factors in Lithuania on the radial growth of *C. orbiculatus* stems and found no specific relationships. The absence of reliable correlations between the width of annual rings and the mean annual temperature, mean winter temperatures, mean summer temperatures, minimum winter temperatures and annual precipitation suggests that climatic conditions in Lithuania are optimal for this species. Surprisingly, even prolonged very cold winter temperatures below –25 °C (in 2003, 2006, 2010, 2012) and occasionally below –30 °C (in 1997) did not damage *C. orbiculatus* shoots significantly to inhibit radial increments in the following growth period. The sites of the three largest and oldest populations studied in Lithuania fall in the first hardiness zone (H1) (Cullen et al. 2011). Thus, areas with moderately cool climates with few periods of severe cold during the winter are suitable for this species and these are areas threatened by its invasion. Beringen et al. (2017) concluded that large areas of Europe might be susceptible to the invasion of *C. orbiculatus* based on an analysis of the climatic conditions in the native range of this species in East Asia and the invasive range in North America. Conditions within the entire Atlantic, continental, and southern boreal biogeographic regions of Europe are likely to be suitable for the establishment of this liana.

Conclusions

Celastrus orbiculatus is spreading in Europe, although its range is still restricted. Climatic conditions are suitable for this species in the Atlantic and continental biogeographic regions, as well as in the southern part of the boreal biogeographic region of Europe. It invades a wide range of forest habitats and poses a threat to dry grasslands, dunes, forest fringe communities of high conservation value, as well as diverse anthropogenic habitats.

Celastrus orbiculatus, at least in Lithuania, but probably also in other European countries, is represented not only by dioecious, but also by monoecious individuals. Thus, even in cases of the occurrence of solitary individuals or small groups of monoecious plants, it produces viable seeds and successfully reproduces sexually. Considering its current distribution and the quite frequent cultivation of *C. orbiculatus* in gardens, most likely it will steadily spread into new areas. Nevertheless, it is still possible to stop the spread of this liana and to reduce the risk of its further invasion.

The effectiveness of control and eradication measures of invasive plant populations is highest when the occupied area is rather small (Blackwood et al. 2010; Epanchin-Niell and Hastings 2010). Control and eradication of *C. orbiculatus* are time- and cost-consuming tasks (Ellsworth et al. 2004a; Pavlovic and Leicht-Young 2011; Beringen et al. 2017). However, considering that total area currently occupied by *C. orbiculatus* in Europe as being ca. 250 ha, its effective control and eradication is still feasible and cost-effective.

Increasing public awareness about the threat of *C. orbiculatus* invasion is among the most important tasks aiming to reduce the possibility of its spread from gardens. Further surveillance of exiting stands of *C. orbiculatus* and search for invaded but still overlooked areas should be performed involving citizens, amateur botanists, and specialists.

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COMMENTARY

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Don't throw the baby out with the bathwater – ban of glyphosate use depends on context

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Recently, Science journal published a letter entitled "Support Austria's glyphosate ban" (Peng et al. 2020) where the authors argue that the usage of glyphosate should cease. They propose that other weed-killing alternatives such as root exudates, crop rotation or mulch be used instead. We agree that risks associated with using this herbicide on a large scale exist, but on a small scale, such as in invasive plants control, glyphosate has an important role and is not easy to replace. Therefore, the context and scale need to be taken into account when applying such bans.

Many aggressively resprouting invasive trees and shrubs, e.g. *Robinia pseudoacacia* (black locust), *Ailanthus altissima* (tree of heaven), or *Acacia* (acacias) can only be effectively eradicated by combining mechanical and herbicide treatments (Dufour-Dror 2013; Krumm and Vítková 2016). Local conditions also limit the use of some methods. Recommended mechanical girdling without herbicide treatment and leaving standing trees to avoid the root resprouting (Vítková et al. 2017) is not suitable for public forests where falling branches may cause serious injuries (Sádlo et al. 2017).

For many herbaceous plants, e.g. *Heracleum mantegazzianum* (giant hogweed), *Fallopia* sp. (knotweeds; syn. *Reynoutria*) or *Rumex alpinus* (monk's rhubarb), the sensible application of herbicides is an effective method of control, because mechanical removal is not feasible (e.g. Bímová et al. 2001; Csiszár and Korda 2017). Such need for herbicide combination with mechanical methods is reported from many regions

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and for many species (e.g. Burn et al. 2003; Nielsen et al. 2007; Csiszár and Korda 2017). A European-wide example is that of knotweeds (*Fallopia* sp.) where mechanical methods include regular cutting with removal of aboveground biomass. Unfortunately, this approach does not lead to eradication; it only lowers the impact and needs to be repeated each year. An alternative option is to dig out the rhizomes which is resource- and labour-demanding if done properly. Both these methods are associated with the need to remove and transport the biomass which can lead to further spread. Spraying with herbicides thus offers much more efficient control of the invaded sites (Jones et al. 2018). In large infestations, the foliar spraying of herbicides is recommended in the first season, followed by spot application or injection to stems or rhizomes. Of course, if foliar spraying is the only option for invasive species management, then selective herbicides should be used as a first option as they allow for faster regeneration of the surrounding vegetation, hence reducing the reinvasion.

The situation is different for annual plant species, such as *Impatiens glandulifera* (Himalayan balsam), where the use of herbicides is not needed as the plants can be destroyed mechanically by pulling due to their weak root system. Such an approach allows for the eradication of large infestations effectively, rapidly and without any side effects to co-occurring biota (Saegesser et al. 2016).

We agree that to minimize the side effects on biodiversity and human well-being, large-scale spraying using a huge amount of herbicides in agriculture needs to be dramatically reduced. However, spot application and other direct methods used for invasive plants control (e. g. hack-and-fill, drill-and-fill, cut-stump) must remain as an option because other effective control methods are currently unavailable.

Herbicides other than glyphosate are very similar in their rate of degradation in soil, mobility and potential abilities to contaminate e.g. water; their effects on nature are comparable and some of them, which might replace glyphosate in the future, have a much less favourable ecotoxicological profile (Burn et al. 2003). It also needs to be considered that a complete ban on glyphosate or similar herbicides might lead to the resignation of some stakeholders regarding the control of invasive species. Thus we call for a balanced approach to the use of herbicides, taking into account the context of an environmental problem in question. Agriculture, as the largest user of herbicides, and nature conservation have different goals that require different methods to achieve. It is necessary to distinguish between application of herbicides for economic reasons such as in large-scale agriculture, and for nature conservation purposes including the control of invasive species representing one of the major threats to biodiversity as identified by many managers (Pyšek et al. 2013).

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RESEARCH ARTICLE

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Monitoring the silver carp invasion in Africa: a case study using environmental DNA (eDNA) in dangerous watersheds

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Abstract

Biodiverse habitats are increasingly subject to an intensification of anthropogenic stressors that may severely diminish species richness. Invasive species pose a dominant threat to biodiversity and biosecurity, particularly in biodiversity hotspots like Kruger National Park, South Africa. The invasive silver carp, *Hypophthalmichthys molitrix*, was introduced into the Olifants River and may experience range spread owing to favorable environmental conditions. Intensive monitoring protocols are necessary to effectively manage invasions of species like silver carp. Unfortunately, tropical and sub-tropical aquatic systems are difficult to monitor using conventional methods (e.g., netting, electrofishing and snorkeling) owing to a range of factors including the presence of dangerous megafauna. Conservation of such systems may be advanced by the adoption of novel methods, including environmental DNA (eDNA) detection. Here, we explore the utility of environmental DNA (eDNA) to conduct safe, reliable and repeatable surveys in dangerous watersheds using silver carp as a case study. We conducted eDNA surveys at 12 sites in two neighbouring watersheds, and determined that the species has expanded its range within the Olifants River and to the

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south in the Sabie River. Expansion in the former is consistent with the presence of suitable spawning conditions. We discuss the implications of this survey for biodiversity monitoring in similar aquatic systems in the tropics and advocate an integrative approach to biomonitoring in these ecosystems.

Keywords

Biomonitoring, hazardous sampling, invasive species, Asian carp, species detection

Introduction

Africa is home to some of the most diverse habitats on the planet, encompassing myriad climatic, geologic and biotic zones (Happold and Lock 2012). This continent contains 22% of the highest-ranked watersheds supporting human populations, 9% of biodiverse hotspots, and is second to only central and southeast Asia in global importance for ecosystem services (Luck et al. 2009). In Africa and beyond, watersheds represent essential components of the socio-economic and cultural landscape, providing key ecosystem services, and are in need of effective and strategic management (Flotemersch et al. 2015).

River systems are threatened by direct modification through channelization (Emerson 1971), channel rerouting, construction of dams, weirs, locks and arterial canal networks, changes in drainage and overflow within the drainage basin (Johnson et al. 2009), and by the translocation of native species and introduction of non-indigenous species (Zhang et al. 2015). Poor watershed management can adversely affect nutrient and habitat availability, species distribution and abundance, population viability, and isolation, erosion or total loss of genetic variation (Davis et al. 2018). Poor management of fisheries and other biotic resources has resulted in introductions of detrimental non-indigenous taxa (i.e. aquatic invasive species; AIS) (Cucherousset and Olden 2011).

Monitoring the biotic component of river systems is essential to effective management of watersheds. For fishes, conventional monitoring of lotic habitats has traditionally relied upon methods including nets (e.g. seine, fyke, gill) or traps, angling, direct observation (SCUBA-diving or snorkeling), electrofishing, and telemetry and acoustic monitoring (Portt et al. 2006). In certain circumstances, however, these methods can be ineffective or too dangerous to deploy. Strong currents can preclude use of these methods or limit their utility to seasonal windows (Portt et al. 2006). Factors idiosyncratic to each method will also limit the scope and utility of each (e.g. low visibility will impact direct observation (Mueller et al. 2006)). In large areas of Africa, as well as Australia, southeast Asia and South America, the presence of large semi- or fully obligate aquatic mammals or reptiles represent a real danger to river researchers and affect surveys by destroying or interfering with equipment (World Health Organization 2003). In addition, the accidental bycatch and mortality of aquatic mammals and reptiles during fish surveys is a growing conservation concern (Ellender et al. 2016; Carrizo et al. 2017).

The Olifants River in southern Africa is home to dangerous aquatic megafauna animals including the common hippopotamus (*Hippopotamus amphibius* (Linnaeus, 1758)) and Nile crocodile (*Crocodylus niloticus* (Laurenti, 1768)) (Carrizo et al. 2017). Safe monitoring of river ecosystems containing all or some of this megafauna is highly problematic using conventional sampling tools. Environmental DNA (eDNA) detection was developed to indirectly detect a target species by collecting cellular and free aqueous DNA shed by the target species into the water column without the need for actual observation or collection of the organism itself (Ficetola et al. 2008; Thomsen and Willerslev 2015). By collecting water, the time and personnel required to conduct sampling are reduced (Thomas et al. 2019), thereby enhancing safety to both surveying personnel and co-occurring wildlife. In addition, accruing evidence indicates that eDNA detection may be more sensitive at detecting rare species than conventional methods (e.g. Dejean et al. 2012; Biggs et al. 2015).

Silver carp (*Hypophthalmichthys molitrix* (Valenciennes, 1844)) was first introduced to South Africa in 1975, when individuals from a German population were donated to the Marble Hall experimental fish farm adjacent to the Olifants River (Lübcker et al. 2014). It was suspected to have spread into the wider Olifants system, including the Olifants River, Lake Flag Boshielo (an impoundment on the Olifants River), and the Massingir dam, Mozambique (Sara et al. 2018), although the extent of its overall distribution remains uncertain. Lübcker et al. (2014) proposed that most of northeastern South Africa was suitable for colonization by this species. Here, we use eDNA to assess presence of silver carp in the Olifants River in Kruger National Park (South Africa) and Limpopo National Park (Mozambique) in southern Africa. We determined the ability to detect silver carp in the system using eDNA and comment on the utility of this method in the field to reduce potential human-wildlife conflict.

Methods

Study organism and sites

Silver carp is a highly invasive fish extensively introduced from its native range in eastern Asia to Europe, North America, and southeast Asia (DeGrandchamp et al. 2007). It is a highly effective filter feeder, consuming a range of microscopic forage that is channeled into high growth and fecundity rates (DeGrandchamp et al. 2007). Experimental and modeling data indicate that many populations of native North American fishes may be extirpated through direct competition or cascading trophic effects resulting from silver carp introduction, thus further spread in southern Africa is worrisome (Zhang et al. 2015).

We identified 12 sites in the Olifants watershed (Table 1; Figure 1) as suitable locations for sampling based upon site access and anecdotal evidence of carp capture, and to determine if a large hydroelectric dam protected the Upper Olifants from be-

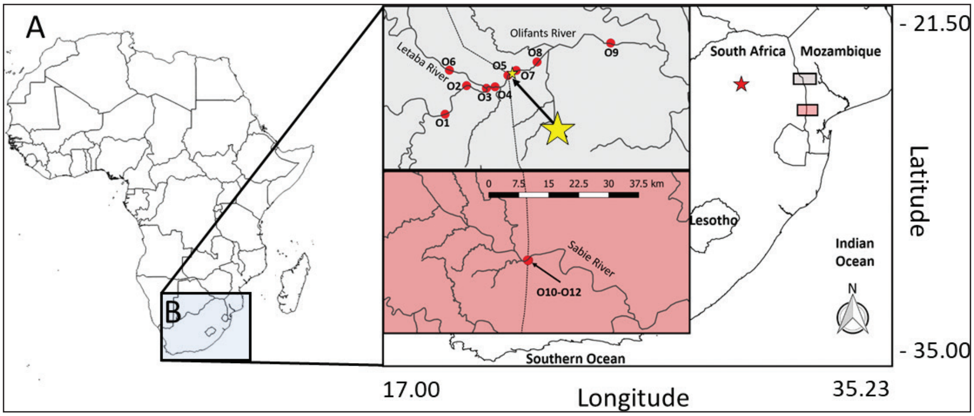

Figure 1. Map of sampling locations for silver carp eDNA within the continent of Africa (**A**). All sampled sites within southeast Africa (**B**) are shown as red circles. The two areas sampled are the Olifants system (Olifants and Letaba Rivers, grey inset) and the Komati (Sabie River, red inset). Also shown is the site of introduction and escape of silver carp in South Africa (red star) and the location of the Massingir dam, Mozambique (yellow star). Dotted line delimits the South Africa – Mozambique border.

Table 1. Sampling information and results of the qPCR analysis for the presence of silver carp eDNA at twelve sites in the Olifants watershed 'PCR' column indicates how many duplicate reactions for each of the three biological replicates were positive. The final column shows the mean Cq values across all positives (omitting non-amplifications) and their standard deviation (SD).

Site Code	Date	Site Description	River	Geographic Co-Ordinates	PCR	Mean Cq ± SD
O1	16/06/15	Olifants River Weir	Olifants	-24.055824, 31.720335	0/0/0	N/A
O2	16/03/15	East of Olifants Camp	Olifants	-23.982616, 31.775594	2/2/0	34.622 ± 0.688
O3	16/03/15	Olifants/Letaba Confluence	Olifants	-23.989445, 31.826483	2/0/1	31.744 ± 0.087
O4	17/03/16	Olifants River Gorge	Olifants	-23.985550, 31.848714	2/2/2	32.434 ± 1.267
O5	17/03/15	South Africa Border	Olifants	-23.956183, 31.881781	0/1/2	34.884 ± 1.737
O6	17/03/15	Letaba River Weir	Letaba	-23.942911, 31.731429	2/2/2	33.809 ± 4.149
O7	19/03/15	Upper Olifants, Above Dam	Olifants	-23.943567, 31.902952	2/0/2	33.914 ± 2.261
O8	19/03/15	Massingir Dam, Pelagic	Olifants	-23.921727, 31.956548	0/2/2	30.615 ± 2.161
O9	20/03/15	Massingir Dam Wall	Olifants	-23.873332, 32.145614	1/0/2	33.549 ± 0.639
O10	21/03/15	Coromana, Mozambique	Sabie	-25.184227, 32.033023	1/2/0	35.038 ± 0.091
O11	21/03/15	Coromana, South Africa	Sabie	-25.185171, 32.031348	2/2/1	34.238 ± 1.619
O12	21/03/15	Upper Sabie, South Africa	Sabie	-25.183838, 32.030184	2/0/1	36.208 ± 2.248

ing invaded from downstream sites. Three sites (O10–O12) were located in the Sabie River, part of the neighbouring Komati watershed, to determine if the carp has spread beyond the borders of the Olifants watershed.

Water collection, transportation and filtration

At each site, three 2 L water samples (3 × biological replicates) were collected in sterile (10% bleach solution (6% w/v sodium hypochloride)) polycarbonate plastic Nalgene bottles. Unless access was difficult, all sampling was conducted by reaching from the bank to extract a sample from the top 5 cm of surface water in the littoral zone. Using single-use gloves for each sampling event, each sterile bottle was swept through the surface until filled. Each sample was immediately placed in a bleach-sterilized cooler and held at 4 °C during transportation back to the laboratory. Where direct access to the riverbank was difficult (e.g., large stretches with high embankments and prolific scrub vegetation), the site was accessed by boat and water was collected from as close to the shoreline as possible. At each site, a single Nalgene bottle containing 2 L distilled water (environment blank control) was opened and exposed to the environment before the top was resealed.

All water samples were filtered immediately upon return from the field in a central bleach-sterilized laboratory located in Skukuza, Kruger National Park, or in an ad-hoc, bleach-sterilized field laboratory near Massingir, Mozambique. Water was vacuum pumped through 1.2 µm pore glass fibre filters (47 mm diameter, VWR 696-filter). The filtration set-up included a tripartite manifold system of three funnels, each provisioned with a magnetic seal that securely clasped a filter between the funnel and the pump. Each biological replicate was filtered simultaneously in each of the three funnels (3 × filters per 2 L sample). After each sample was filtered, the entire apparatus and surrounding area was bleached sterilized, wiped with distilled water, and left to dry before proceeding with the next sample. After each filtration event, a separate set of sterile forceps was used to submerge each filter in a 2 ml Eppendorf tube containing 95% ethanol for storage at -20 °C. All samples were shipped to Canada for eDNA detection analysis.

eDNA extraction

eDNA extraction was performed in a dedicated extraction space. Using a protocol adapted from Dougherty et al. (2016), filters were cut into quarters and placed in tubes containing 20 µl 1 mm-diameter glass beads and 500 µl of modified (Coyne et al. 2005) CTAB (2% w/v cetyltrimethylammonium bromide, 2% w/v polyvinylpyrrolidone, 1.4M NaCl, 100mM Tris-HCL, 20nM EDTA) buffer pre-warmed to 65 °C in a heat block. Samples were homogenized using an unequal gravity FastPrep F120 homogenizer (Thermo Savant Instruments, Ltd) at 6.5 m sec^{-1} for two minutes and then incubated at 65 °C for two hours. 500 µl chloroform-isoamyl alcohol was added to each tube, mixed over-end for 5 minutes and centrifuged at 13,000 g for 15 minutes. The upper aqueous phase was transferred to a new tube containing 500 µl ice-cold isopropanol and 250 µl NaCl solution to precipitate out the DNA whilst incubating at -20 °C overnight. After incubation, the tubes were centrifuged again at 13,000 g for 15 minutes to allow DNA to pellet at the bottom. The supernatant was discarded and 200 µl ethanol wash added prior to vortexing and centrifugation at 13,000 g for three minutes. A second ethanol-washing round followed prior to carefully discarding the ethanol and drying the pellet in a vacufuge at maximum spin before being centrifuged for 10–15 minutes at 35 °C. The DNA pellet was resuspended in 100 µl 1X T.E. buffer and heated to 55 °C for ten minutes. After each filter had been extracted from the filter paper, the eluted DNA from each quarter filter paper was pooled into a single tube before decanting into 50 µL aliquots to be stored long-term at -20 °C.

eDNA quantitative real-time polymerase chain reaction (qPCR) detection

The Asian carp invasion in North America has resulted in development of numerous eDNA assays to detect all four problem species, including two in the genus *Hypophthalmichthys* (Jerde et al. 2013). Because only silver carp has been known to have been introduced into the Olifants River and surrounding systems, we chose the assay with the highest sensitivity to detect fish in the genus *Hypophthalmichthys* (Wozney and Wilson 2017). We chose the CTM primer set (Carp Taqman Multiplex 1), without using the Taqman probe, to increase sensitivity, designed and optimized in singleplex reactions (Wozney and Wilson 2017) to amplify both species. The original intent of this assay was to amplify both target species but discriminate based on differential probe binding (see Wozney and Wilson 2017 for assay specifications).

All qPCR reactions were performed in a laboratory with no previous history of Asian carp tissue or DNA samples. For each pooled eDNA extract, two duplicate technical replicates (PCR reactions) were performed. For all 12 sites, this tallied to 72 reactions in total, six per site/location and two per biological replicate. Alongside the target reactions (and environmental blank – one per location), two no-template controls were run to control for qPCR reagent/sample contamination. All reactions were performed on a single reaction plate, thereby eliminating inter-run variance in PCR results. All reaction volumes were 20 µl, consisting of 200 nM of each primer (0.4 µl), 10 µl of PowerUp SYBR green mastermix (Applied Biosystems, USA) and the remaining volume (9.2 µl) made up of eDNA extract providing the template for the reaction. To determine whether PCR inhibition may impede positive detection, we reassessed each sample using a separate internal positive control (IPC) assay that consisted of a primer and probe set that amplify a unique, and not found in nature, manufactured DNA sequence. We used the Taqman-probe based IPC developed by Gasparini et al. (2020) in which 200 copies of the artificial template were added to every reaction alongside 9.2 uL of the eDNA sample with Taqman Universal Mastermix II (Applied Biosystems, USA) in lieu of PowerUp SYBR Green. Inhibition was inferred to have occurred if the PCR Cq values were delayed by more than 1 Cq value relative to the Cq value of NTC control (i.e. pure water added as template). qPCR was performed on a 96-well CFX Thermal Cycler (BioRad) and run according to the published protocol (Wozney and Wilson 2017). PCR data were recorded in two ways: 1) successful amplification defined as the production of an amplification curve that passed a threshold of fluorescence; and, 2) of those reactions that amplified successfully, the Cq (quantification cycle) value, whose quantity is inversely proportional to the amount of silver carp DNA in the original sample, was documented.

Statistical analysis

We initially performed a post-hoc power analysis to confirm that the sampling design was sufficient to correctly reject the null hypothesis of no detection, thus boosting confidence in any negative finding. We used Olson et al.'s (2012) method:

$$Y_{i,j} = \Pi_{i=1}^{j}(1-\hat{d})$$

where y = probability of a false negative (upper boundary of 0.05 (alpha)), j = number of samples to be subject to qPCR, and $\hat{d}$ is the proportion of samples (out of three biological replicates) that yield ≥ 1 eDNA positive qPCR detection, to determine the predicted minimal number of samples necessary to achieve 95% power, predicated on our observed data.

Following the adoption of eDNA as a proxy of occupancy in habitat occupancy models (e.g. Schmidt et al. 2013), we performed a simple estimation of detection probability (p), site occupancy (Ψ) and the probability of detecting silver carp eDNA per sampling event (θ). As eDNA sampling is inherently hierarchical, Bayesian models predicting the three detection parameters are less vulnerable to increasing variability in the data than frequentist methods (Doll and Lauer 2014). Although occupancy modeling is most useful when co-estimating variables that may influence the detection of the organism by its proxy (i.e. via eDNA molecules), hierarchical models that estimate base values of each of the above parameters would result in more accurate values than would a naïve calculation based on naked data alone (Dorazio and Erickson 2018). To estimate these parameters, we performed the analysis using the R package ‘eDNAoccupancy’ (Dorazio and Erickson 2018) employing 500,000 Markov Chain Monte Carlo iterations after which model parameters were fitted (discarding first 10% as burn-in). Finally, an analysis of variance of Cq value across sites and by river system, which were encoded as categorical variables, was performed using the ‘aov’ function in R.

To assess if the Olifants River is suitable for silver carp spawning, a preliminary assessment was completed using methodology developed by Heer et al. (2019). The assessment determined whether a tributary is suitable to potential Asian carp spawning using a decision tree that categorized the river into four categories (not suitable, minimally suitable, moderately suitable, and highly suitable). It was based on growing degree-days (base 15 °C; GDD15) accumulated in a 12-month period spanning 2017–2018, an estimated hatching distance, and water temperature and velocity thresholds for spawning. There were five thresholds: 633 GDD15 within a year to achieve maturation, minimum water temperature of 17 °C to initiate spawning run, estimated distance to hatch is less than the unimpounded length of the river, flow velocity spike of 0.7 m/s to initiate spawning, and 900 GDD15 to trigger mass spawning. To complete the assessment, mean daily water temperature was obtained for the Mamba weir and mean daily velocity was estimated at the Balule weir based on weir dimensions and measured stage and discharge data. One year of temperature and velocity data were available, July 1, 2017 to June 30, 2018.

Results

Molecular eDNA detection

Every site except for O1 was positive for silver carp eDNA in at least one technical replicate across three biological replicates (Table 1). Apart from site O1, all locations were positive for silver carp eDNA and had detection levels ≥ 50 % (at least three out of six technical PCR replicates). Sites O4 and O6 had 100% detection across the board, although they did not have the lowest mean Cq value, which occurred at site O8 (Mean Cq = 30.62 ± 2.16). Overall mean Cq across all successful PCRs and all sites was 33.63 ± 2.39. All samples, apart from O3, O9 and O10, did not show qPCR inhibition as all IPC Cq values were within the margin of acceptability (i.e. all samples were within 1 Cq of the mean NTC IPC value of 30.63). In these cases, a PCR negative could be correctly interpreted as a lack of target eDNA molecules in the template volume. However, for sites O3, O9 and O10, the IPC did not amplify at all (Cq = 0), indicating complete inhibition of the IPC.

Detection analysis

The percentage of successful biological replicates yielding at least one detection ($\hat{d}$) was 69.40 %. Post-hoc power analysis revealed that the minimal predicted number of biological replicates per site should be two for this system, assuming 95% power, thus providing confidence that non-detection of silver carp at site O1 was unlikely due to a lack of appropriate sampling intensity. An occupancy analysis resulted in a high level of detection of silver carp in the study area. Estimated global probability of detection (p) was 0.849, inferred site occupancy (Ψ) was 0.892, and sample detection probability (θ) was 0.754. An analysis of variance indicated a near-significant effect of watershed and sampling site upon the levels of silver carp eDNA (F-value = 2.910, p = 0.0657; and F = 2.005, p = 0.0652, respectively) (Figure 2 and Suppl. material 1: Table S1). However, two-way ANOVA revealed that watershed was more important in determining silver carp occupancy than individual site effects (F-value = 3.298, p = 0.0494 and F-value = 1.682, p = 0.140 for River and Site, respectively). The concentrated aggregate of sampling sites on the more southerly Sabie River tended to have higher mean Cq values – and thus lower quantity of silver carp eDNA – than most of those in the northerly Olifants system in South Africa (Figure 2).

Spawning habitat analysis

Preliminary spawning assessment indicates that the Olifants River was a minimally appropriate habitat in 2017–2018 (Figure 3). The river was always above the minimum 17 °C temperature, but never achieved flow sufficient to reach the higher levels of suit-

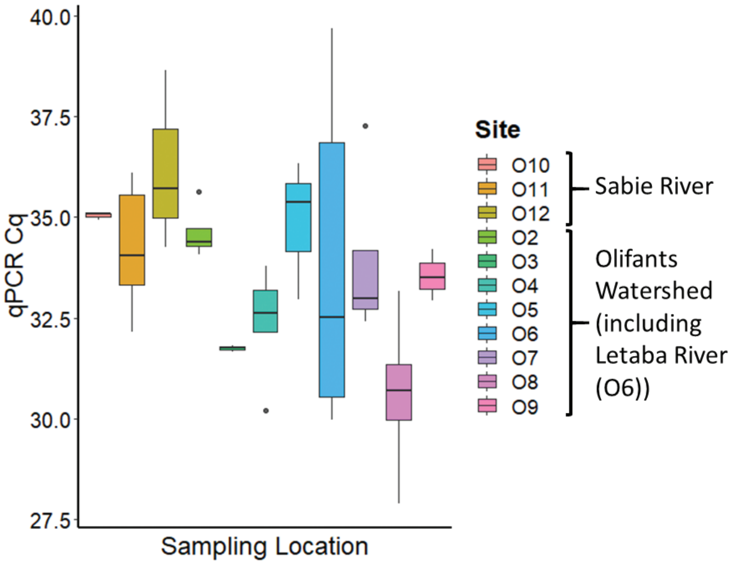

Figure 2. Levels and variance of qPCR detection (y-axis) of silver carp DNA among sampling sites within and between rivers in the Olifants system and Sabie River (x-axis).

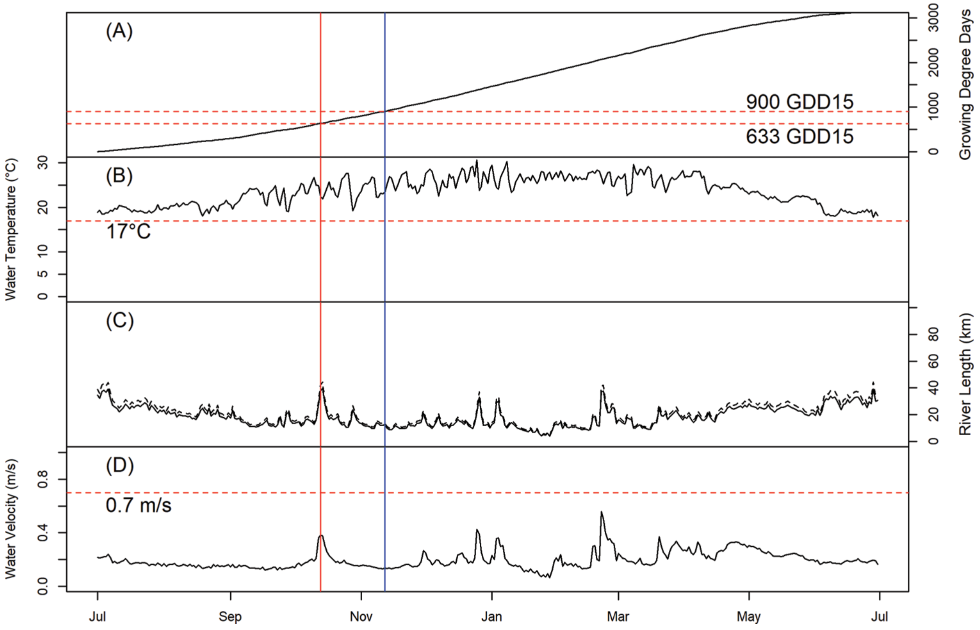

Figure 3. Results of a silver carp spawning suitability model for the Olifants River, July 1, 2017-June 30, 2018 **A** growing degree days (base 15). 633 GDD15 (red dashed line and solid red vertical line) is required for maturation and 900 GDD15 (blue dashed line and solid blue vertical line) is required for initiation of mass spawning **B** mean daily water temperate. Minimum temperature required (red dashed line) is always exceeded **C** unimpeded river length required for egg hatching to occur **D** mean daily water velocity. Flow spike required for high suitability is not achieved.

ability. Based on a July 1 winter start date, 633 GDD15 would have been achieved by early October and 900 GDD15 by early November. Following these spawning initiation dates, the length of unimpeded river required for egg hatching ranged 15–40 km, which is readily available in the Olifants River.

Discussion

Silver carp range expansion as inferred from eDNA presence data

Environmental DNA detection confirmed the presence of silver carp eDNA throughout the sampled areas of the Olifants River and neighbouring systems (where sampled), except for Site O1. Our study adds to the mounting evidence confirming this technique as a sensitive tool to monitor highly invasive Asian carps generally (Jerde et al. 2011, 2013; Turner et al. 2015; Wozney and Wilson 2017) and silver carp in particular (Amberg et al. 2015; Coulter et al. 2019; Stepien et al. 2019). Our study is the first to use a targeted eDNA approach to monitor for the presence of Asian carps on the African continent. However, the detection of silver carp was not without impediment, as the IPC results indicate that, in some sections of the Olifants and Sabie rivers, low levels of eDNA were beyond the level of sensitivity of the assay. The IPC was set at 200 copies per reaction, so even slight inhibition was detected. As many eDNA species targets are rare and shed low levels of eDNA, PCR inhibition is a potentially huge issue for delineation of the invasion front and detection of rare species where low abundances should translate into low eDNA levels and, possibly, false negatives. However, as we observed 3/6 silver carp qPCR detections for each of the IPC inhibited sites, we can conclude that, although silver carp eDNA levels were lower than at the other sites (except O1), they often exceed 200 copies per 9.2 μL (qPCR template volume) and are thus well within the level of detectability of this assay. As PCR inhibitors are present in the systems of study, it is likely that silver carp Cq values were delayed, if not outright inhibited, and thus it would be difficult to translate Cq values to copy numbers. Moreover, silver carp eDNA levels exhibit a nonlinear relationship with carp density (Coulter et al. 2019).

eDNA detection levels were high (except Site O1), with at least half of all qPCR reactions positive for silver carp. Notwithstanding the likely nonlinear relationship between eDNA levels and population densities, the fact that silver carp was detected in half of all qPCR reactions at three sites for which the IPC (200 copies per reaction) was completely inhibited is consistent with a large, established population constantly shedding a lot of eDNA into the surrounding environs. These results indicate that silver carp is pervasive in the Olifants watershed, supporting the proposition that this species is established within South Africa (Ellender and Weyl 2014). The data confirm the expansion of the silver carp range into Kruger National Park from elsewhere in the Olifants system, either from upstream at the Flag Boshielo Dam, Mpumalanga (Britts 2009) or from downstream (Schneider 2003). The data also confirm that the Massingir population is still extant and healthy and corroborates local reports of the successful fishing of this species for local consumption. It is unlikely that escapees, or the intermittent use of silver carp as a baitfish, would yield a similar pattern of strong detections. All field controls were negative, thereby discounting the possibility of spurious results stemming from contamination as a factor responsible for positive detection results.

One criticism of eDNA utility in lotic systems, however, is the potential systemic bias introduced by the directional flow of water. Until recently, the conveyance of eDNA downstream was thought to operate primarily on relatively small spatial scales (up to 12 km) varying by hydrology, season and target organism (e.g. Deiner and Altermatt 2014; Shogren et al. 2017). Interestingly, Pont et al. (2018) reported eDNA transport distances of over 100 km in the voluminous River Rhône, France. However, if all positive eDNA tests in the upper Olifants River resulted from advection from a single upstream source, one would expect the eDNA signal to attenuate (e.g. show rising Cq values) with increasing distance from that source, albeit at low flow conditions (Jane et al. 2015). We observed no such trend with our data. The silver carp eDNA signal was strong above and below Massingir Dam, a large reservoir of the Olifants River on the border of South Africa and Mozambique. This is consistent with Schneider (2003), who reported silver carp invading the Massingir Dam in 1996/1997 and dispersing downstream into the mainstream Limpopo River during floods in 2000 (Schneider 2003). Natural dispersal events may also be supplemented by accidental and intentional anthropogenic release of larval and juvenile carp, which are hard to identify during early stages of ontogeny and which may be used as baitfish. In the Great Lakes region of North America, juvenile silver carp have been positively identified using qPCR as a part of the selection of baitfish available to anglers (Stepien et al. 2019).

Affirmation of habitat suitability for establishment of silver carp

Due to the long unimpounded distance of the Olifants River upstream of Massingir Dam, along with high temperatures, this system is suitable for silver carp spawning. Temperature data in the river indicate that maturation can occur by early October and mass spawning by early November based on a July 1 winter start date. However, silver carp maturity can be reached within approximately three months, indicating that spawning could potentially occur at any point in the year. The minimum temperature required for spawning (17 °C) was always met in this system. There exists some uncertainty with this approach, primarily due to the estimate of velocity based on weir dimensions and the assumption of linearity in the hatching distance.

Our findings are consistent with those derived from ecological niche modelling, in which the middle-lower Olifants River was assessed to have a high predicted climatic suitability for silver carp establishment (>75%) (Lübcker et al. 2014). However, the Komati watershed lies in an area predicted to have 80–100% suitability for the establishment of the silver carp. The modelling result is consistent with our observation of eDNA in the Sabie River, albeit at lower levels than in the Olifants. Interestingly, silver carp has yet to be visually observed here, thus eDNA detection would be the first record of its occupancy in the Sabie River. The discrepancy in eDNA levels between the two watersheds is likely a function of the time since invasion. Silver carp has occupied the Olifants River at least since escaping captivity in the upper reaches of the river in 1992 (Britts 2009). Assuming the eDNA signal to be redolent of occupancy of silver carp in the Sabie River, it may be that the species has yet to establish and expand its range, although such an event may be anticipated if invasion follows a stepping-stone model of expansion (Alharbi and Petrovskii 2019). Indeed, the large floods caused by Cyclone Eline (UNDP 2000) resulted not only in the flooding of several fish farms containing silver carp in the lower Limpopo River floodplain in Mozambique, but also linked the Limpopo and Komati Rivers in March 2000 (Schneider 2003).

eDNA detection as a tool for aquatic biomonitoring in dangerous systems

The adoption of eDNA detection methods is especially pertinent in areas in which conventional monitoring can be inefficient or dangerous. These areas are often in the tropics, and harbor much of the world's biodiversity, including charismatic freshwater megafauna (Carrizo et al. 2017). At the time of our survey (2015), we adopted best practices for that time. This included taking physical water samples from the surface of water in close proximity to a range of unpredictable predators (Nile crocodile) and large herbivores (hippopotamus), which, while not without risk, was safer than deploying and collecting nets or physically entering the water. This also reduced the risk of accidental by-catch and accidental mortality of conservation priority taxa such as Nile crocodile, which are susceptible to entanglement and drowning in sampling gear such as gill nets (Ellender et al. 2016). As such, using eDNA to successfully detect the silver carp validates this approach for use in dangerous systems. Using eDNA lowers the risk of loss of equipment and time to pernicious and unpredictable events in the field. Dangerous systems may not just be limited to those containing large animals but also those with high levels of pollutants or pathogens, strong tidal or convectional currents, or rapids and unpredictable, seasonal flows. In the intervening time period, onsite eDNA developments have progressed so rapidly that much of the physical workflow is automated and can be operated remotely, including performing onsite qPCR detection (Thomas et al. 2018, 2019). Onsite methods also have the advantage of reducing the risk of laboratory-based contamination and the degradation of rare eDNA molecules in transit (attenuating both Types I and II error). Thus, eDNA detection methodology is suitable for highly biodiverse and remote areas such as Kruger National Park.

The success of this pilot project to investigate the utility of using eDNA detection to identify the presence of the silver carp in two watersheds in southern Africa cannot be disputed, at least for AIS. However, at-risk species (SAR) show similarities with invasion-front species in that often their *a priori* distribution is unknown or merely suspected, and whose populations are fragmented and characterized by few individuals. Yet, eDNA has been shown to be just as effective at targeting SAR as it has AIS (e.g. Balasingham et al. 2018; Currier et al. 2018). Furthermore, the molecular methods used to conduct eDNA monitoring may also be extended. For example, qPCR-based tools can also be used to screen eggs to confirm spawning success in suitable habitat identified by *a posteriori* modelling or previous eDNA detection (Fritts et al. 2019).

Conclusion

We recommend that eDNA detection be used as part of the conservation biologist's toolbox when considering the management of invaders in dangerous aquatic ecosystems in the tropics and elsewhere. Moreover, future investigations should take into account the complexities of hydrodynamics when monitoring rivers, potentially by using hydrodynamic models (e.g. Garcia et al. 2013), which could be integrated with eDNA data (e.g. Carroro et al. 2018). Our results indicate that the Olifants River is suitable for silver carp spawning and could reach high suitability if it coincided with a flow spike that exceeds 0.7 m/s. These projections were validated by the eDNA detection data. We highlight the combined power of eDNA detection and habitat modelling tools to predict not only current distribution – and habitat suitability – of a target organism, but to also forecast which areas are at risk of imminent invasion. Adopting an integrative methodology, combining aspects of molecular, theoretical and field ecology to better effectively manage extremely limited resources is beneficial to optimizing efforts to conserve important refuges of aquatic biodiversity in the coming decades.

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Supplementary material 1

Table S1

Authors: Steven Crookes, Tej Heer, Rowshyra A. Castañeda, Nicholas E. Mandrak, Daniel D. Heath, Olaf L.F. Weyl, Hugh J. MacIsaac, Llewellyn C. Foxcroft

Data type: analytical table

Explanation note: ANOVA of eDNA detection levels (Cq) across sites and rivers.

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RESEARCH ARTICLE

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Biogeographical comparison of terrestrial invertebrates and trophic feeding guilds in the native and invasive ranges of *Carpobrotus edulis*

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Abstract

Plant invasions impact on biodiversity by altering the composition of native communities by disrupting taxonomic and functional diversity. Non-native plants are often released from their natural enemies, which might result in a reduction of the attack of primary consumers. However, they can also be exposed to the attack of new herbivores that they might not be able to tolerate. Hence, invertebrate communities can be influenced by invasive non-native plants, which in turn modify interactions and change environmental conditions. In this study, we examined the compositional and trophic diversity of invertebrate species, comparing ecosystems with and without the plant species *Carpobrotus edulis* in coastal areas in its native (South Africa) and introduced (Iberian Peninsula) ranges. Results show that *C. edulis* has a clear impact on invertebrate communities in its non-native range, reducing their abundance in invaded areas, and particularly affecting certain trophic groups. Invasive *C. edulis* also alters the invertebrate diversity by not

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only reducing abundance but also by altering species composition. Overall, the physical dominance of *C. edulis* modifies the co-occurrence of invertebrate assemblages, reducing the number of trophic groups and leading to substantial effects on primary consumers. Results suggest that the lack of natural enemies might be an important driver of the expansion of *C. edulis* in its introduced range. Further work is needed to examine long-term changes caused by non-native plants on invertebrate assemblages and the subsequent modification of biological interactions.

Keywords
Alien species, beta-diversity, biological invasions, enemy release hypothesis, insects, invasion ecology, plant-animal interactions, species richness

Introduction

Drivers of change such as globalization, habitat fragmentation, and climate change facilitate the arrival, establishment and proliferation of invasive non-native species (Rossman 2009; Litchman 2010; Early et al. 2016). Biological invasions may induce a wide variety of environmental impacts, such as biodiversity loss (Habel et al. 2019) or functional changes of the invaded ecosystems (Vitousek et al. 1997). Many native organisms, including soil biota and assemblages of plants and animals, can be directly or indirectly threatened by invasive species (McCary et al. 2016; Smith-Ramesh 2017; Wardle and Peltzer 2017). One reason why invasive species can induce such impacts is because they can occupy the functional space of natives (Loiola et al. 2018), leading to novel biotic interactions in resident communities (Prior et al. 2015; van Kleunen et al. 2018). Some authors have pointed this out for invasive plants, highlighting that they can alter invertebrate assemblages through non-trophic mechanisms (McCary et al. 2016; Smith-Ramesh 2017; Rodríguez et al. 2020) resulting in ecosystem-wide cascading effects (Sugiura et al. 2013; López-Núñez et al. 2017). Many studies have explored how plant invasions alter biotic interactions in invaded ecosystems (Traveset and Richardson 2020), but there is little research on the changes in biotic interactions mediated by invasive plants comparing native and introduced ranges (DeWalt et al. 2004; Maron et al. 2004; Callaway et al. 2011). In this regard, biogeographical studies comparing impacts of invasive plants in their native and invasive ranges can help us to elucidate whether the magnitude of impacts is enhanced in the introduced range (Hierro et al. 2005).

Non-native plants are often released from their natural enemies (i.e. Enemy Release Hypothesis; Keane and Crawley 2002) but can also accumulate many native herbivores in the introduced range (Maron and Vilà 2001; Levine et al. 2004), which may affect native invertebrate communities in different ways. While some studies have reported that plant invasions can lead to dramatic declines of native insect diversity, others have shown that diversity does not decrease because the native biota is replaced by other non-native or native invertebrate species (Hejda et al. 2017). Sakai et al. (2001) suggested that functional and taxonomic groups may interact in ways that protect the composition of some communities against invasion more than others. Hence, the impacts of invasive plants on invertebrate assemblages may vary from negative to neutral or positive depending on their role in the community (Litt et al. 2014; Clusella-Trullas and Garcia 2017). Moreover, non-native plants can alter the feeding behaviour of only certain invertebrate assemblages through non-trophic mechanisms (Procheş et al. 2008; Smith-Ramesh 2017). Some authors have reported that non-native plants may induce strong negative effects on primary (McCary et al. 2016) and secondary consumers (Langellotto and Denno 2004; Scherber et al. 2010). Notwithstanding these findings, other authors have reported that predators may benefit from plant invasions (Gomes et al. 2018). For pollinators, effects vary, depending on the studied species (Davis et al. 2018). Increases in the amount of organic matter contributed by invasive plants rarely benefit detritivores (Castro-Díez and Alonso 2017). Hence, to understand the impacts of invasive plants on native invertebrate communities, it is important to consider groups of invertebrates with diverse functional, behavioural and trophic roles. Nonetheless, most studies on the impacts of plant invasions have focused only on particular assemblages of arthropod species (Spafford et al. 2013; Bezemer et al. 2014).

Plant invasions are a major concern in Europe (European Union 2014; European Commission 2017), where the South African mat-forming succulent *Carpobrotus edulis* (L.) N.E. Br. (Aizoaceae) is among the most problematic invasive plant species (Nentwig et al. 2018). This perennial clonal plant (for details see Gonçalves 1990) inhabits cliffs, rocky shorelines and dunes in South Africa, and now threatens a wide range of Mediterranean-climate coastal areas in Australia, California, Chile, France, Italy, Portugal and Spain (D'Antonio and Mahall 1991; Traveset et al. 2008; Vilà et al. 2008; Campoy et al. 2018). *Carpobrotus edulis* was intentionally introduced to Europe for gardening in the 17th century, and it was subsequently deliberately disseminated for sand and dune stabilization (Campoy et al. 2018). Its capacity for clonal propagation allows *C. edulis* to spread horizontally (Roiloa et al. 2010; Fenollosa et al. 2016), and to form dense monospecific stands that can cover the encircling surface in a few years (D'Antonio and Mahall 1991). This can lead to changes in the dynamics of the invaded areas by replacing local flora (Novoa et al. 2012; Novoa et al. 2013) and altering the invertebrate communities (Bartomeus et al. 2008; Rodríguez et al. 2019). *Carpobrotus edulis* represents a severe threat for the conservation of coastal ecosystems in the Iberian Peninsula (Campoy et al. 2018), and the factors influencing its invasion and its impacts are well documented (Novoa and González 2014; Roiloa et al. 2014; Lechuga-Lago et al. 2016; Souza-Alonso and González 2017). However, little attention has been given to its influence on the species composition and trophic groups of native invertebrate communities (Rodríguez et al. 2019). Moreover, most studies of *C. edulis* related to invertebrate communities have only targeted the herbivorous feeding guild (Rodríguez et al. 2018; Vieites-Blanco et al. 2019). Therefore, it is necessary to study the direct and indirect effects of *Carpobrotus* on the trophic and taxonomic groups of native invertebrates in invaded ecosystems (Spafford et al. 2013; Bezemer et al. 2014).

We investigated the composition and trophic feeding guilds of invertebrate species by comparing coastal areas with and without *C. edulis* in the native (South African) and introduced (Iberian Peninsula) range of the species. We hypothesized that (i) areas with

C. edulis will show higher differences in diversity compared with areas where the species is absent in its non-native range, the Iberian Peninsula. We also hypothesized that (ii) the composition of invertebrate species will be altered through reduced beta-diversity in invaded areas due to the lower replacement of species. We also expected (iii) a higher diversity of trophic groups in well-preserved areas in the native range of *C. edulis*.

Methods

Study area

The study was carried out at ten locations, five of them within the native range of *C. edulis* in the Cape Floristic Region (Western Cape, South Africa) and five in the introduced range in the north-western Iberian Peninsula (Galicia, Spain, and Região Norte, Portugal) (Suppl. material 1: Table S1). The study areas share similar characteristics such as the Mediterranean sub-humid climate with Atlantic trend (Beck et al. 2018) and coastal dune vegetation (Allsopp et al. 2014; González et al. 2017). To ensure independence in the invertebrate community, locations in both regions were separated by at least 15 km. In each study area, dominant plant species, vegetation cover and distance to the sea were recorded (Suppl. material 1: Table S1). Sampling was conducted in 2016 during spring, the flowering season for both native and introduced ranges (Campoy et al. 2018), coinciding with the time period of maximum emergence of most invertebrates in nymphal and adult forms (from March to June, in the Iberian Peninsula and from September to December, in South Africa).

Sampling design

We established two different sampling areas at each location: plant communities with and without *C. edulis*, with an area of 200 m^2 each (20 m × 10 m) and at least 100 m apart ($n = 5$). To compare the invertebrate occurrences between areas by visual spot-checks (i.e. hand collection by inspecting each plant), we randomly dropped a standard quadrat grid (0.5 m × 0.5 m) at 3 m intervals ten times within each plot. To identify and quantify the number of aerial invertebrates we used a total of 100 yellow sticky traps (0.2 m × 0.1 m; two faces) in late spring (June in the Iberian Peninsula and December in South Africa). Five traps were evenly spaced within each plot (between 0.2 m to 0.5 m above ground) (see Fig. 1 for details) and were maintained for a week. All invertebrates in the specific adult life form found in each quadrat and collected by the traps were sorted and assigned to a morphospecies using morphological differences (Oliver and Beattie 1996). Each individual was then identified to a taxon group (order, family, genus and species or morphospecies) using invertebrate field guides (Chinery 1997; McGavin 2002; Barrientos 2004; Picker et al. 2004; Ruiz et al. 2006) and the assistance of expert entomologists (Suppl. material 1: Table S2).

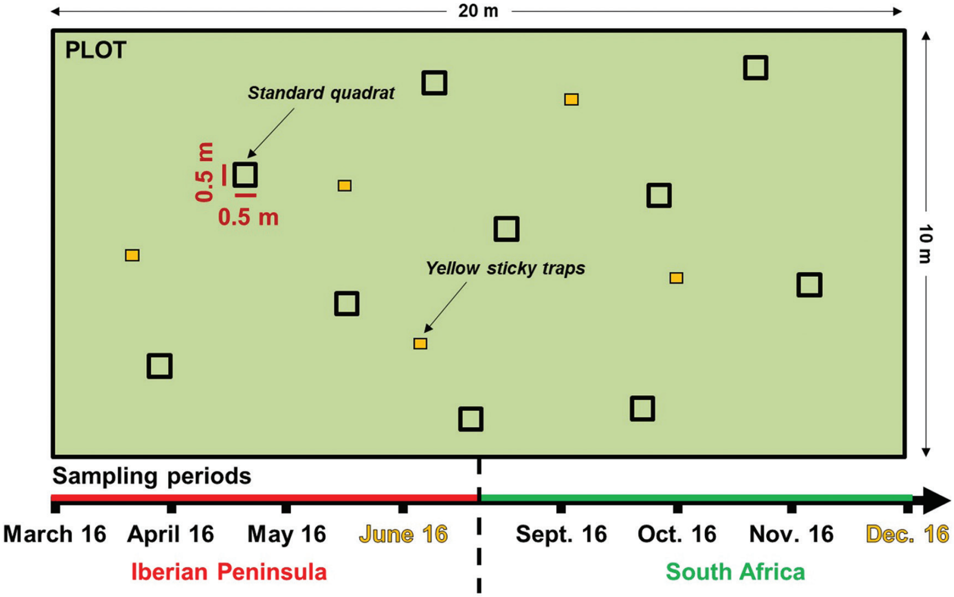

Figure 1. Schematic representation of the experimental design for assessing differences in terrestrial invertebrates and trophic feeding guilds in the native (South Africa) and invasive (Iberian Peninsula) ranges of *Carpobrotus edulis*. Within the plot, standard quadrats (black squares) and yellow sticky traps (yellow rectangles) are represented.

Based on their mouthparts and the predominant feeding behaviour of each identified family/genus/species, we assigned each individual to a trophic feeding guild, roughly corresponding to different trophic levels: detritivores, herbivores, nectar feeders, parasitoids and predators.

Data analysis

We estimated the invertebrate species richness between the coastal areas of South Africa and the Iberian Peninsula that differ in plant community (with and without *C. edulis*) by using the *specpool* function in the 'vegan' R package v.2.5–2 (Oksanen et al. 2018). To establish sampling representativeness, we used four non-parametric species estimators (Chao2, Jack 1, Jack 2 and bootstrap), taking into account that invertebrate assemblages usually have rare species (Hortal et al. 2006). Species accumulation curves were used to represent the cumulative species as a function of the number of plots sampled to examine the accumulative and rarefaction number of species (Gotelli and Colwell 2001), and the species diversity was then compared between areas using sample-size-based rarefaction and extrapolation (R/E) curves (Chao et al. 2016). We computed species accumulation curves using the *specaccum* function in 'vegan' R package. Sample-size- based (R/E) curves were applied to quantify three measures of species diversity (Hill numbers) with standardised sample size, including unconditioned 95% CI using the 'iNEXT' R package (Hsieh et al. 2016).

To evaluate the differences in the invertebrate community, we used Generalized Linear Mixed Models (GLMMs) to test the effect of the region (South Africa and Iberian Peninsula) and plant community (with and without *C. edulis*) for differences in invertebrate species abundance, richness and diversity indices (Margalef, Shannon, Simpson and Pielou evenness). We compared invertebrate trophic and taxonomic diversity between areas using GLMMs to test the effect of the region and plant community for differences on abundance and species richness. To establish statistical comparisons between models, we used the *glmer* function in 'lme4' R package v.1.1–19 (Bates et al. 2015). Models were carried out with species nested within the plant community using Wald Chi-square Method and maximum likelihood (Laplace Approximation) using Poisson distribution and link function = log, while comparisons between areas were examined using the 'lsmeans' R package (Lenth 2016).

To examine beta-diversity differences, we used a multivariate test for homogeneity of dispersion analyses (PERMDISP) of differences in invertebrate composition among studied areas. We assessed the species turnover (replacement of one species by others among locations of the same plant community), nestedness (species richness gain or loss among locations where species lists vary across different plant communities) and total beta-diversity (Anderson 2006; Baselga 2010). PERMDISP analyses determined the average distance of observation to the geometric centre (centroid) of each predefined group, e.g. invertebrates associated with *C. edulis* from the Iberian Peninsula (Anderson 2006). Significance tests were carried out based on a Jaccard's dissimilarity matrix and 999 permutations using the *beta.pair* function in 'betapart' R package (Baselga and Orme 2012).

Lastly, the species composition was analysed using a non-metric multidimensional scaling (NMDS) ordination based on a Bray-Curtis similarity matrix of standardised and log(x+1) transformed data. Differences were tested using a permutational multivariate analysis of variance (PERMANOVA) with the *adonis* function (strata = location) in 'vegan' R package. PERMANOVA was used to test for differences among coastal areas of South Africa (n = 5) and the Iberian Peninsula (n = 5) for the effect of plant community (with and without *C. edulis*), distance to the sea, vegetation cover, and their interaction. All statistical analyses were performed using the software programme R (R Development Core Team, 2019; v.3.6.1).

Results

Alpha-diversity

A total of 13,785 invertebrate individuals were identified by visual spot-checks and yellow sticky traps; these were assigned to 90 families and 170 morphospecies (Suppl. material 1: Tables S2–S3). Species belonged to 19 distinct orders, of which six orders

(Araneae (12 species), Coleoptera (17), Diptera (61), Hemiptera (32), Hymenoptera (20) and Lepidoptera (9)) and six feeding guilds (detritivores (13), herbivores (58), nectar feeders (15), omnivores (19), parasites (20) and predators (45)) were used for the data analysis. The remaining orders were grouped into a category named "Other taxa" (13); these included Amphipoda, Coccoidea, Collembola, Isopoda, Julida, Mantodea, Neuroptera, Opiliones, Orthoptera, Psocoptera, Stylommatophora, Thysanoptera and Trombidiformes. Relative abundances of invertebrate groups differed between the studied areas (Fig. 2). In South Africa, the relative abundances in areas with *C. edulis* tend to be more balanced among groups, where we found a higher abundance of other taxa and Formicidae than in areas without *C. edulis* (Fig. 2). For the Iberian Peninsula, our results showed a reduction of Diptera and Other taxa at areas with *C. edulis* (Fig. 2). However, we found an increase for Hemiptera and Hymenoptera at areas with *C. edulis*.

Table 1. Number of collected invertebrate species (S_{obs}) and individuals as well as the estimated richness of species (calculated by Chao2, Jack 1, Jack 2 and bootstrap species estimators) from South Africa and Iberian Peninsula at locations that differ in plant community (with and without *Carpobrotus edulis*).

Area	S_{obs}	Individuals	Chao2 (±SD)	Jack1 (±SD)	Jack2	Bootstrap (±SD)
Overall (all combined)	171	13785	210.86 (17.47)	207.63 (7.35)	227.40	187.87 (4.06)
Overall South Africa	104	8283	123.64 (11.32)	124.58 (5.82)	134.39	113.65 (3.29)
Carpobrotus edulis	89	4684	105.28 (9.34)	109.16 (7.08)	117.02	98.66 (3.95)
No *Carpobrotus edulis*	91	3599	110.53 (10.73)	113.08 (6.20)	122.78	101.26 (3.29)
Overall Iberian Peninsula	94	5502	124.11 (15.87)	119.48 (6.06)	134.09	105.30 (3.23)
Carpobrotus edulis	75	2283	100.13 (13.70)	98.04 (6.86)	110.42	85.32 (3.66)
No *Carpobrotus edulis*	72	3219	118.46 (29.51)	93.12 (6.28)	108.95	80.87 (3.26)

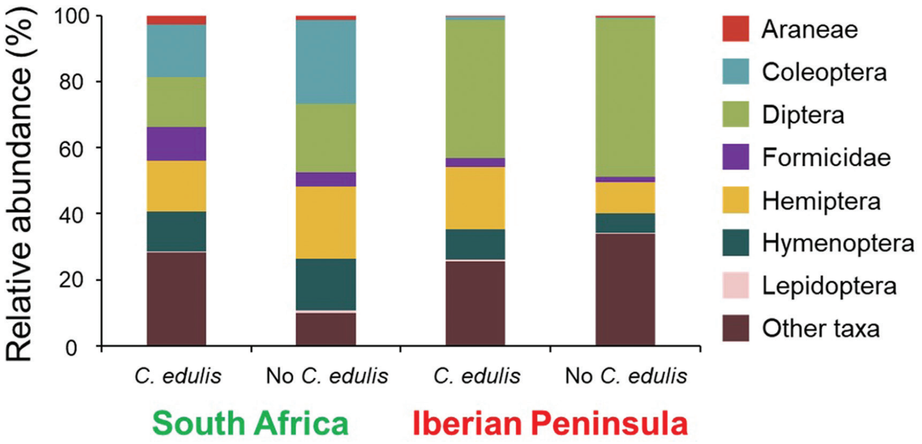

Figure 2. Relative abundances of taxa (order) within each studied area. The abundance of each taxon was calculated as the percentage of sequences per location for a given invertebrate group. The group 'Other taxa' encompasses grouped orders with lower abundance.

Species accumulation curves and the estimated number of species in relation to the species observed indicated that the sampling effort was adequate to capture the majority of the species (Suppl. material 1: Fig. S1; Table 1). Overall, species richness estimators showed that the coastal areas of South Africa had the highest quantity of observed and estimated species, despite no large differences having been observed for estimated species between regions (Table 1). Sample-size-based rarefaction and extrapolation (R/E) curves showed differences among the areas (Fig. 3), where we found values close to saturation for the exponential of Shannon's entropy index and the inverse of Simpson's concentration index. Nevertheless, we did not find low slopes towards the end for species richness. For South Africa, our results showed a higher species diversity in areas without *C. edulis* than in areas where the species is present (Fig. 3). The results are different for the Iberian Peninsula, where the invaded areas had generally higher species diversity estimates (Fig. 3). Comparing the two regions showed that areas with *C. edulis* overlapped when using the 95% confidence intervals for diversity estimates for species richness and Shannon diversity, while substantial differences were found in areas without *C. edulis* (Fig. 3).

GLMMs indicated that the levels of abundance and invertebrate diversity indices (species richness, Margalef, Shannon, Simpson, Pielou evenness) tended to be signifi-

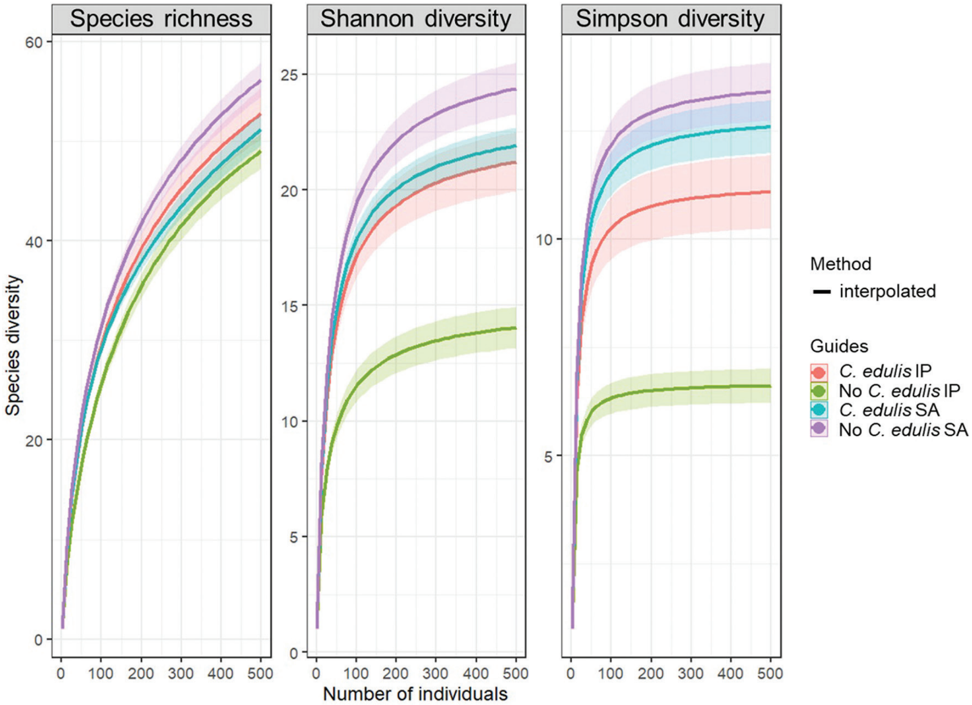

Figure 3. Sample-size-based rarefaction (solid line segment) sampling curves with 95% confidence intervals (shaded areas) for the invertebrate species richness of areas with and without *Carpobrotus edulis* for both regions, South Africa (SA) and Iberian Peninsula (IP), separated by diversity order: species richness (left panel), Shannon diversity (central panel) and Simpson diversity (right panel).

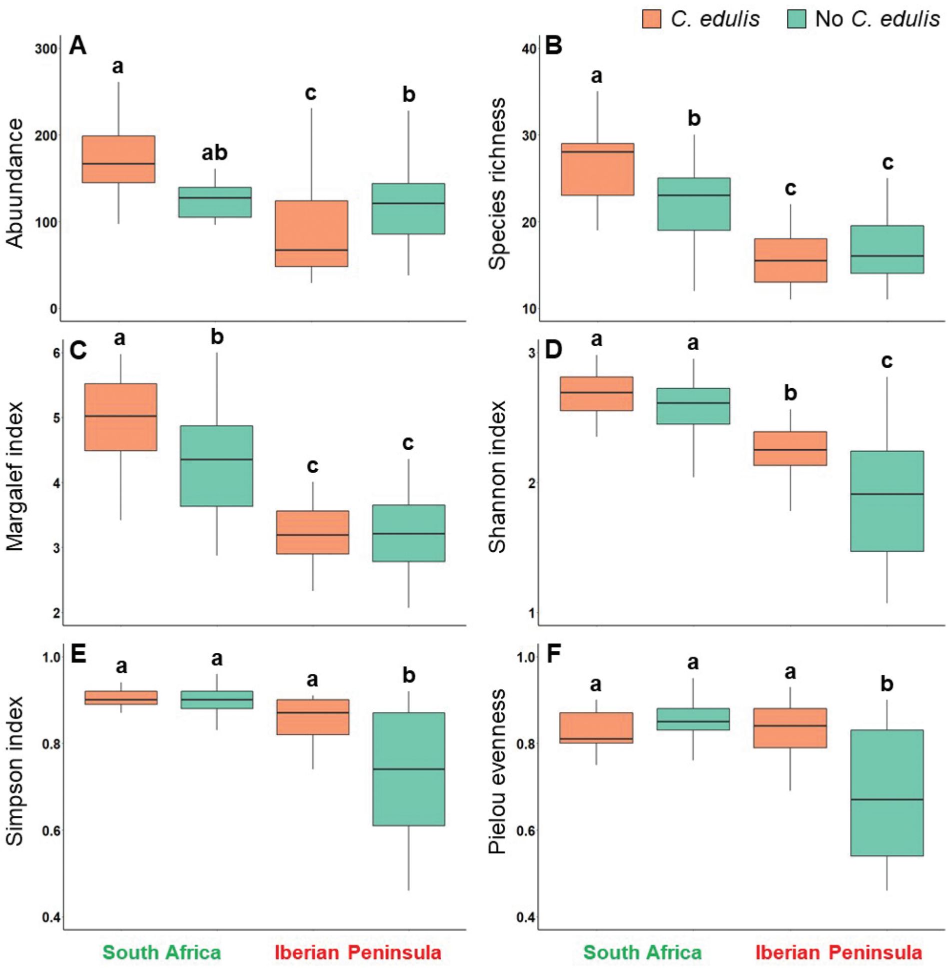

Figure 4. Effect of the plant community (with and without *Carpobrotus edulis*) for differences in **A** abundance **B** species richness **C** Margalef **D** Shannon **E** Simpson and **F** Pielou Evenness recorded among regions of South Africa and Iberian Peninsula. Model-adjusted least square means values ± SE are shown. Different letters indicate statistical significance at $p \leq 0.05$ level using Generalized Linear Mixed Models.

cantly higher in South Africa than in the Iberian Peninsula (Fig. 4; Table 2). Our results showed significant differences for the interaction of region and plant community, in abundance and diversity indices (Table 2). On average, the areas with *C. edulis* in South Africa had higher values of abundance, species richness, and Margalef and Shannon indices (about 100%, 70%, 50% and 20% more, respectively) than the areas with *C. edulis* in the Iberian Peninsula (Figs 4A–D). However, for the Simpson and Pielou evenness indices, no significantly different values were found between South Africa and the Iberian Peninsula in the areas with *C edulis*. The values of these indices were, however, significantly lower in areas without *C. edulis* in the Iberian Peninsula than in any of the other studied areas (Figs 4E–F).

Table 2. Results from the Generalized Linear Mixed Models (GLMM) to test the effect of the region (South Africa and Iberian Peninsula) and plant community (with and without *Carpobrotus edulis*) for differences in abundance, species richness and diversity indices (Margalef, Shannon, Simpson and Pielou evenness) between the invertebrate samples collected. Models were carried with species nested within plant community using Wald Chi-square Method and restricted maximum likelihood (REML). Abundance and species richness were fitted by maximum likelihood (Laplace Approximation) using Poisson distribution and link function = log.

Effect	df	χ^2	Pr(>Chisq)
Abundance			
Region (R)	1, 100	4.717	**0.029***
Plant community (PC)	1, 100	0.903	0.342
R × PC	1, 100	377.912	**0.001*****
Species richness			
Region (R)	1, 100	18.851	**0.001*****
Plant community (PC)	1, 100	4.021	**0.044***
R × PC	1, 100	7.661	**0.005****
Margalef index			
Region (R)	1, 100	14.824	**0.001*****
Plant community (PC)	1, 100	11.956	**0.001*****
R × PC	1, 100	6.085	**0.013***
Shannon index (H')			
Region (R)	1, 100	15.014	**0.001*****
Plant community (PC)	1, 100	13.294	**0.001*****
R × PC	1, 100	5.587	**0.018***
Simpson index (D)			
Region (R)	1, 100	7.618	**0.006****
Plant community (PC)	1, 100	18.651	**0.001*****
R × PC	1, 100	17.924	**0.001*****
Pielou evenness (J)			
Region (R)	1, 100	3.959	**0.046***
Plant community (PC)	1, 100	13.053	**0.001*****
R × PC	1, 100	32.060	**0.001*****

df = Degrees of Freedom, *Pr(>Chisq)- p-value*. *Asterisks* indicate statistical differences *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Values in bold indicate significance at $p \leq 0.05$.

Beta-diversity

PERMDISP analyses revealed no dissimilarity for species turnover, nestedness and total beta-diversity, indicating that beta-diversities are very similar among areas with and without *C. edulis* (Fig. 2; Suppl. material 1: Table S4). In South Africa, the invertebrate species composition was only altered by the distance to the sea and its interaction with plant community (Fig. 5A; Table 3A). The NMDS ordination, which fit the data with two axes (Stress = 0.22, Fig. 5A), demonstrated that distance > 500 m is positioned above and distance 0–500 m below on the axis NMDS 2. In addition, the ordination separated the area with *C. edulis* at the left and without *C. edulis* at the right on the axis NMDS 1. Nevertheless, we did not find significant differences that support this representation. In the Iberian Peninsula, the PERMANOVA results showed that the

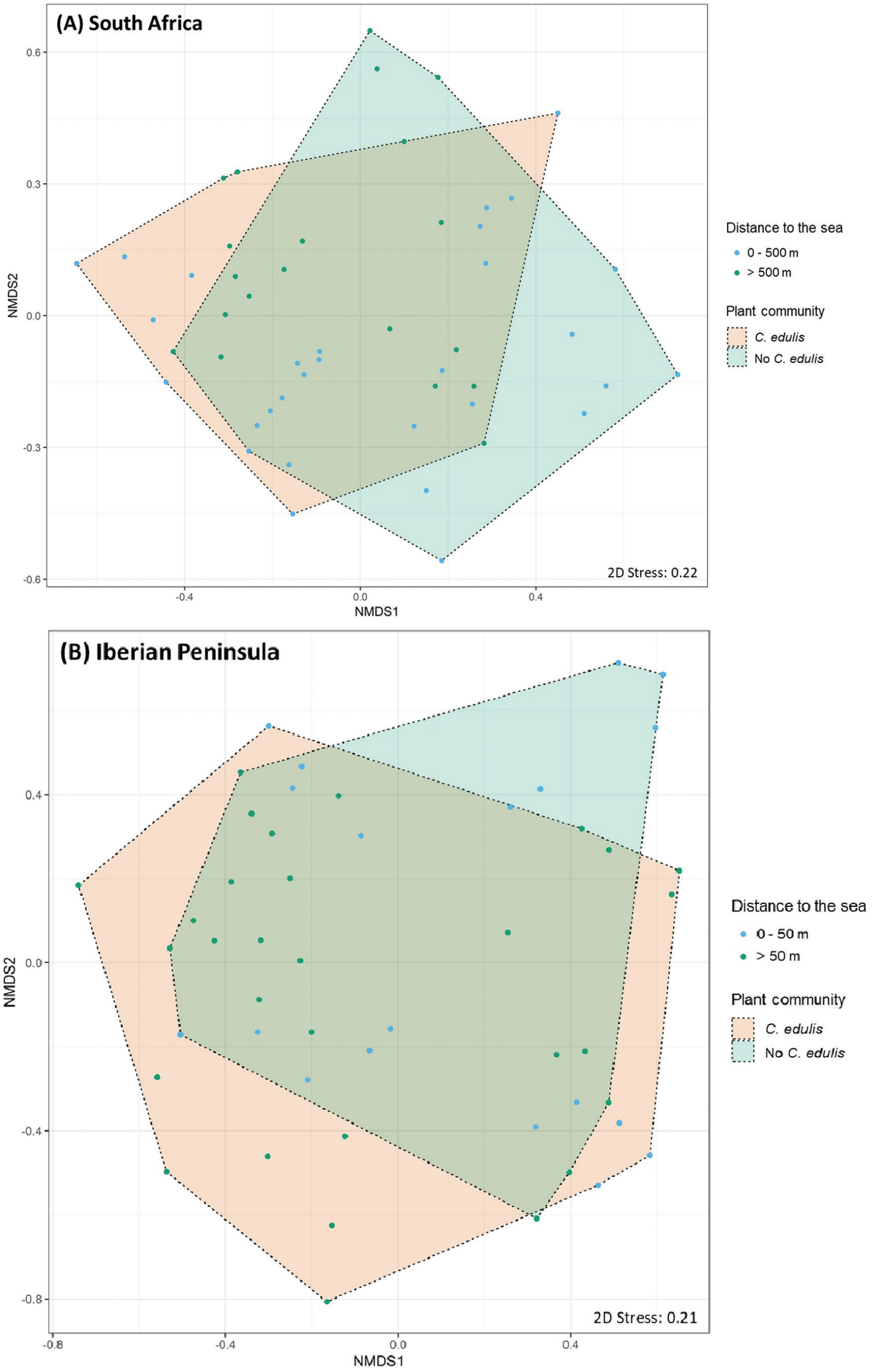

Figure 5. Non-metric multi-dimensional scaling based on a Bray–Curtis matrix of dissimilarities in **A** South Africa and in **B** Iberian Peninsula, showing distances between areas for the species composition and differences in the plant communities (with and without *Carpobrotus edulis*) and distances to the sea.

Table 3. Results from the multivariate permutational analysis (PERMANOVA) of differences for South Africa and Iberian Peninsula at locations that differ in plant community (with and without *Carpobrotus edulis*), distance to the sea and vegetation cover. The PERMANOVA with the *adonis* function (strata = location) in the 'vegan' package in R was based on a Bray-Curtis similarity matrix of standardised on log(x + 1) transformed data.

PERMANOVA	df	SS	MS	*Pseudo-F*	R^2	*Pr(>F)*
(A) South Africa						
Plant community (PC)	1	0.095	0.095	0.767	0.014	0.413
Distance to the sea (DS)	1	0.388	0.388	3.121	0.056	**0.001*****
Vegetation cover (VC)	1	0.227	0.227	1.824	0.032	0.403
PC × DS	1	0.418	0.418	3.365	0.060	**0.001*****
PC × VC	1	0.140	0.140	1.124	0.020	0.446
DS × VC	1	0.254	0.254	2.040	0.036	0.131
PC × DS × VC	1	0.198	0.198	1.593	0.028	0.190
Residuals	42	5.224	0.114		0.752	
Total	49	6.945			1.000	
(B) Iberian Peninsula						
Plant community (PC)	1	0.300	0.2997	1.744	0.031	**0.032***
Distance to the sea (DS)	1	0.248	0.2485	1.445	0.026	**0.002****
Vegetation cover (VC)	1	0.284	0.2843	1.654	0.030	0.336
PC × DS	1	0.758	0.7577	4.408	0.080	**0.001*****
PC × VC	1	0.109	0.1096	0.638	0.011	0.864
DS × VC	1	0.249	0.2491	1.449	0.026	0.343
PC × DS × VC	1	0.338	0.3386	1.970	0.035	**0.027***
Residuals	42	7.219	0.1792		0.759	
Total	49	9.507			1.000	

df = Degrees of Freedom, SS = Sum of Squares, MS = Mean Square, *Pseudo-F*- *F*-statistic, *Pr(>F)*- *p-value*. *Asterisks* indicate statistical differences *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$. Values in bold indicate significance at $p \leq 0.05$.

invertebrate species composition was altered by the presence of *C edulis* (i.e. plant community), the distance to the sea and their interaction, but not by vegetation cover (Fig. 5B; Table 3A). The NMDS ordination, which fit the data with two axes (Stress = 0.21, Fig. 5B), showed that the areas with *C. edulis* are positioned along the axis NMDS1 and below on the axis NMDS2, while those without *C. edulis* are positioned mainly above on the axis NMDS2. Similar results were found for distance to the sea, where distance > 50 m is below, and distance 0–50 m is above on the axis NMDS 2.

Trophic and taxonomic groups

GLMMs revealed significant differences for almost all the groups (except detritivores), while for species richness significant differences between plant communities and regions were only found for herbivores, nectar feeders, parasites and predators (Suppl. material 1: Table S5). Abundance of almost all trophic groups was significantly higher in areas with and without *C. edulis* in South Africa, while the areas with *C. edulis* in the Iberian Peninsula had the lowest values, except for herbivores (Suppl. material 1: Table S5). Besides, the areas with *C. edulis* in South Africa had the highest values for species richness, while areas with and without *C. edulis* in the Iberian Peninsula tend to have the lowest values, except for detritivores and omnivores (Suppl. material 1: Table S5).

Within taxonomic groupings, results revealed a significantly higher abundance of almost all groups in areas with and without *C. edulis* in South Africa (except Diptera), while the areas without *C. edulis* in the Iberian Peninsula had the lowest values, except for Araneae, Diptera and Other Taxa (Suppl. material 1: Table S5). Similar results were found for taxonomic species richness, where the areas with *C. edulis* in South Africa had highest values, except for Diptera and Lepidoptera. By contrast, the significantly lowest values were found in the Iberian Peninsula; for Araneae, Formicidae and Hemiptera in areas with *C. edulis* and for Coleoptera, Hymenoptera, Lepidoptera and Other taxa in areas without *C. edulis*. (Suppl. material 1: Table S5).

Discussion

Alpha-diversity

The Cape Floristic Region of South Africa is recognised as a global biodiversity hotspot (Myers et al. 2000). Moreover, it is known that some non-native plants can offer suitable habitat for diverse assemblages of arthropods (Prasad and Hodge 2013; Rodríguez et al. 2019). Nevertheless, non-altered areas without introduced plants tend to support more diversity than invaded areas (Bezemer et al. 2014; van Hengstum et al. 2014). We therefore initially hypothesized that areas with *C. edulis* will show higher differences in diversity compared with areas without it in its non-native range, the Iberian Peninsula. As predicted, our results demonstrate that coastal areas in South Africa had higher values for the abundance and diversity indices (species richness, Margalef, Shannon, Simpson and Pielou evenness) than the areas in the Iberian Peninsula. However, in the Iberian Peninsula, we found that the presence of *C. edulis* significantly reduced only the abundance, while it increased the values of the Shannon, Simpson and Pielou evenness indices. Some authors have noted that introduced plants may induce a negative effect on the arthropod communities (Procheş et al. 2008; Dibble et al. 2013; Van der Colff et al. 2015; Maoela et al. 2016). However, this does not seem to be the case for *C. edulis* (Rodríguez et al. 2019). Although *C. edulis* clearly has negative impacts on native ecosystems in the Iberian Peninsula (Novoa et al. 2012; Novoa et al. 2013), our results show an increase in terms of invertebrate diversity indices.

Biodiversity is not always reduced following invasion of non-native plants. Invasive plants can induce the replacement of the native biota by species with similar characteristics (Hejda et al. 2017). Hence, alterations to biodiversity caused by invasive plants can affect native arthropod assemblages and their trophic role in the community in many ways – neutrally, negatively or even positively (Litt et al. 2014; Clusella-Trullas and Garcia 2017). Our results showed that species estimators and rarefaction curves in coastal areas of South Africa had the highest quantity of observed and estimated species, and were higher for areas without *C. edulis*. However, the opposite patterns were found for the Iberian Peninsula. This can be explained by the fact that few rare non-native species can live exclusively in areas where *C. edulis* is present in the Iberian Peninsula (Rodríguez et al. 2019); this can influence the species estimations due to their low abundance. There is evidence of insect declines in heavily plant-invaded areas (Habel et al. 2019; Richard et al. 2019), and the effects of invasive plants on recipient communities can be predictable and preventable (Buckley and Catford 2016). However, our results showed no decline in local richness of native invertebrates in invaded areas. In some cases, non-native plants can favour the presence of native generalist species, but they can also promote a considerable increase of non-native insects (Wingfield et al. 2011; Crous et al. 2016; Cordero-Rivera et al. 2019). Therefore, these results might be explained by the fact that the invasion does not change alpha diversity over time (Pandolfi and Lovelock 2014), because species gains could compensate species losses (species turnover) due to an increase in beta-diversity (Dornelas et al. 2014). Therefore, the changes can seem small or be otherwise unapparent to date, but disruptive effects of invasive plants sometimes turn out to be important for rare insects (Wagner and Van Driesche 2010).

Beta-diversity

Plant invasions frequently alter the structure of native communities by disrupting their trophic interactions and changing the ecological processes of the invaded areas, which often creates new environmental scenarios (Crous et al. 2016; Rodríguez et al. 2019). It is therefore expected that the presence of *C. edulis* modifies the distribution and presence of invertebrates in its invasive range (i.e. the Iberian Peninsula), but not in its native range (i.e. in South Africa). Hence, our second hypothesis proposes that the composition of invertebrate species will be altered by reducing beta-diversity in invaded areas due to the lower replacement of species. As expected, our results showed that invertebrate species composition was affected by the presence of *C. edulis* in the Iberian Peninsula, while we did not find differences in South Africa. However, contrary to our expectations, we found no differences in beta-diversity for all studied areas. This is probably explained by the fact that *C. edulis* is chemically not so different from the neighbouring plants and is, therefore, “edible”. Another possible explanation relates to the direct effects of decreased plant diversity (Litt et al. 2014). Many plant invasions alter the habitat structure inducing severe impacts on ecosystems (McCary et al. 2016; Smith-Ramesh 2017), but consequences of the invasion process are species- and habitat-specific (Richardson et al. 2007). Studying the effect of introduced plants invading different microhabitats is key to detecting an alteration of the associated native fauna (Harvey et al. 2014). On the other hand, our results showed that the distance to the sea alters the species composition, suggesting that salt spray and/or plant diversity, or even prevalent wind (Wiktelius 1981; Nguyen and Nansen 2018), could mediate the diversity of invertebrate species in areas closer to the sea. This finding agrees with the results of Rodríguez et al. (2019) which showed that the invertebrate species composition varies with distance to the sea, with beta-diversity tending to be higher in areas further from the coastline.

Trophic and taxonomic groups

We expected a higher diversity of trophic groups in well-preserved areas in the native range of *C. edulis*. Accordingly, in the Iberian Peninsula, the abundance and species richness of trophic groups were significantly lower than in South Africa. Moreover, our results show that, in the Iberian Peninsula, the abundance and species richness of feeding guilds tended to be significantly reduced by the presence of *C. edulis*. These results agree with previous research which found that introduced plants can alter the trophic diversity in invaded areas (Procheş et al. 2008; Moroń et al. 2009; Maoela et al. 2016; McCary et al. 2016; Rodríguez et al. 2020). The reduction in the abundance of herbivores, omnivores and predators in the areas invaded by *C. edulis* compared with those without *C. edulis* is striking. In agreement with our results, introduced plants can have strong negative effects on primary consumers (McCary et al. 2016). Procheş et al. (2008) also noted that herbivorous insects, but not other insects, are scarce on non-native plants. This agrees with the Enemy Release Hypothesis (ERH) (Elton 1958; Keane and Crawley 2002), which suggests that invasive non-native species are favoured by the loss of their natural enemies in the introduced range, enhancing their vigour by the reduction of pressure from natural enemies (Jeffries and Lawton 1984; Colautti et al. 2004; Ceryngier et al. 2018). Non-native plants can also favour the increase of secondary consumers as predators (Van der Colff et al. 2015; Smith-Ramesh 2017; Gomes et al. 2018). However, our results showed that the invasive plant *C. edulis* can reduce the presence of predators, as also happens with other invasive plants (Langellotto and Denno 2004; Scherber et al. 2010). Nevertheless, the reduction in natural enemies is not necessarily constant over time (Flory and Clay 2013; Crous et al. 2016; Stricker et al. 2016). Invasions are dynamic and invasive non-native species could acquire enemies over the years after their introduction (Hawkes 2007; Schultheis et al. 2015). Thus, monitoring is necessary to confirm that we are not overestimating the alteration of the native invertebrate communities (i.e. long-term sampling intervals).

At the taxonomic level, our results show differences mainly between regions, but also the invasive *C. edulis* in the Iberian Peninsula significantly influences the abundance of Diptera, Hemiptera and other taxa groups. In areas invaded by *C. edulis*, the reduction of secondary consumers as predators could imply a reduction in predation pressure on herbivorous invertebrates (members of Hemiptera and Coleoptera) (Simao et al. 2010), increasing the possibility of herbivore damage to the introduced plant. However, our results also show a reduction in the abundance of Diptera and Other taxa in invaded areas by *C. edulis* in the Iberian Peninsula.

Ecosystem processes and species functional diversity at a local scale can be influenced by the invasion of non-native plants (Simao et al. 2010). Combining the use of trophic and taxonomic diversity is advantageous for improved comprehension of the impacts of invaders on ecological processes (Gomes et al. 2018). An increase in the extent and abundance of non-native plants can result in decreased trophic diversity, even if local native diversity is maintained (Vellend et al. 2013; Dornelas et al. 2014). By contrast, other authors have noted that non-native plants tend to support higher invertebrate taxonomic diversity than native plants, whereas native assemblages have more specialist species, resulting in higher diversity (Okimura and Mori 2018). Hence, maintaining trophic diversity is key to preserve the link between biodiversity and ecosystem functioning (Isbell et al. 2011; Veen et al. 2018). Our results show that trophic and taxonomic dissimilarity was mainly determined by the replacement of species and functions between regions and the areas with and without *C. edulis*. These findings reinforce the interpretation that the invasion of *C. edulis* in the Iberian Peninsula alters the composition of invertebrate species and trophic groups (Rodríguez et al. 2019).

Conclusion

Overall, coastal areas in South Africa are more diverse and had a greater abundance of invertebrates compared to the Iberian Peninsula. Invertebrate species composition was affected by *C. edulis* in the Iberian Peninsula, while the results did not indicate the same in South Africa (see Fig. 6). Therefore, the invasive non-native plant *C. edulis* alters invertebrate assemblages and trophic groups in invaded areas, which may modify plant-invertebrate interactions. *Carpobrotus edulis* has a strong negative effect on primary consumers, suggesting that the lack of natural enemies might be key to its expansion in its introduced range. Understanding whether non-native species cause ecological harm or benefit over species at different community levels is crucial, especially to provide insights to guide conservation and restoration programmes. To this end, further work is needed to examine long-term changes caused by invasive plants on invertebrate assemblages and the subsequent modification of ecological networks.

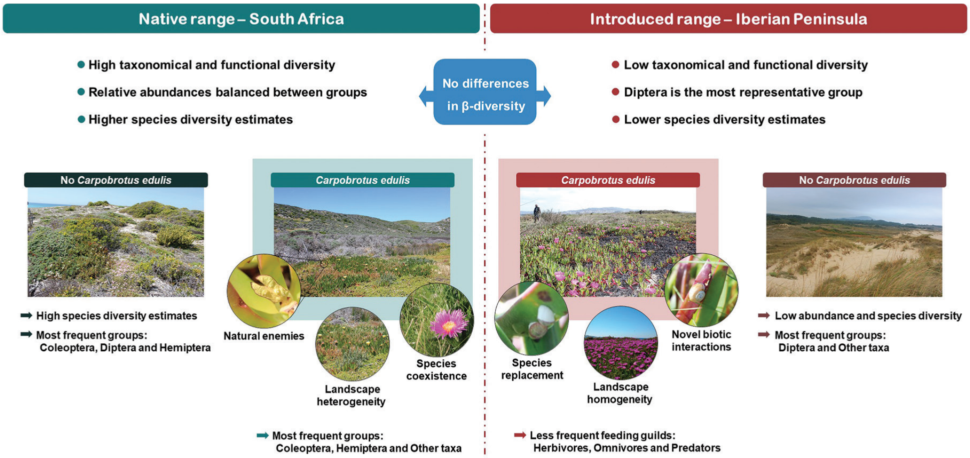

Figure 6. Theoretical diagram describing the main results obtained comparing ecosystems with and without the plant species *Carpobrotus edulis* in coastal areas in its native and introduced ranges. On the left side, the native range is represented (Western Cape, South Africa). On the right, the invasion of *C. edulis* causes changes in the invaded areas (Galicia, Spain).

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Supplementary material 1

Biogeographical comparison of terrestrial invertebrates and trophic feeding guilds in the native and invasive ranges of *Carpobrotus edulis*

Authors: Jonatan Rodríguez, Ana Novoa, Adolfo Cordero-Rivera, David M. Richardson and Luís González

Data type: Studied areas, characteristics, occurrences, tables, images

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SHORT COMMUNICATION

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Do dense layers of invasive plants elevate the foraging intensity of small mammals in temperate deciduous forests? A case study from Pennsylvania, USA

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Abstract

Monospecific stands of invasive plants can dramatically restructure habitat for fauna, thereby elevating population densities or promoting foraging of consumer species who benefit in the altered habitat. For example, dense stands of invasive plants may protect small mammals from predators, which in turn could increase foraging pressure on seeds that small mammals feed upon. We used a before-after, control-impact experimental design to test whether small mammal capture rates were higher and giving-up densities (GUDs) lower beneath dense stands of *Berberis thunbergii*, an invasive shrub with a rapidly expanding range throughout eastern North America. Our experimental design included three plot categories: 1) plots heavily invaded by *B. thunbergii*, 2) control plots lacking invasive shrub cover, and 3) invaded plots where we eradicated *B. thunbergii* midway through the study. Although our overall small mammal capture rate was low, small mammal captures were 65% higher in *B. thunbergii* invaded habitat relative to control plots and eradication lowered capture rates by 77%. GUDs were also 26% higher within *B. thunbergii* relative to control plots and eradication decreased GUDs by 65%. Our findings suggest that small mammals perceive dense stands of *B. thunbergii* as relatively safe foraging habitat. Prior surveys within our study locations revealed dramatically depressed tree seedling densities under *B. thunbergii*, thus invasive plants may promote intensive foraging by small mammals and reduce recruitment for species with foraged seeds or seedlings.

Keywords

Berberis thunbergii, foraging, giving-up density, recalcitrant understory, small mammals

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Introduction

Nonnative plant species that become invasive frequently form dense, nearly monospecific layers that can cause substantial declines in native plant species abundance and diversity. One mechanism that may underlie these declines is that invasive species may commonly provide privileged foraging areas, whereby vertebrate seed and seedling predators gain refuge from their enemies (Orrock et al. 2009; Orrock et al. 2015; Guiden and Orrock 2017). Thus, seed predation by small mammals may be much greater beneath dense layers of invasive plant species than in nearby uninvaded patches. Consequently, any reduction in the diversity and abundance of native plant species could be caused by habitat-mediated predation rather than interspecific resource competition between exotics and natives (e.g., Royo and Carson 2005, 2008; Durra et al. 2011). For example, both experimental (Dutra et al. 2011) and observational (Mattos et al. 2010) studies have demonstrated that dense layers of the invasive Amur honeysuckle (*Lonicera maackii*) protected small mammals from predators, thereby resulting in elevated foraging event frequencies.

Habitat-mediated predation may be a common phenomenon throughout eastern North American forests because many common invasive plant species are understory shrubs or small trees known to form dense stands (see reviews by Royo and Carson 2006; Richardson and Rejmánek 2011). Common invasive trees or shrubs include several species of honeysuckle (*Lonicera* spp.), glossy buckthorn (*Rhamnus cathartica*), Chinese privet (*Ligastrum sinense*), autumn olive (*Eleagnus umbellata*), burning bush (*Euonymus alatus*), and Japanese barberry (*Berberis thunbergii*) among several others. Elevated seed predation has been well-investigated beneath dense stands of native species, however, whether dense understory layers of invasive shrub species typically create preferred habitats for small mammals remains unclear because there are too few studies to draw any firm conclusions (Orrock et al. 2015). Moreover, non-native species may lead to declines in small mammals if they provide fewer seed resources compared to the native species they displaced. Here, we focus on one of the most widespread and pernicious invaders, Japanese barberry, and evaluate whether barberry creates privileged foraging grounds across three different urban forests using a rigorous BACI experimental design (see below).

Japanese barberry (*Berberis thunbergii*, hereafter referred to as barberry) is a woody shrub native to East Asia that represents a key model species for investigating cascading effects of invasive, shrubs. Barberry was introduced to North America in the late 19th century and has spread to at least 32 U.S. states and 6 Canadian provinces (USDA, NRCS 2017). The shrub often forms dense stands that cause both structural and functional shifts in forest understories (Clark and Seewagen 2019). Recalcitrant barberry stands typically reduce temperatures and increase humidity, soil pH, and available soil nitrate relative to uninvaded areas (Kourtev et al. 1999, 2003; Williams and Ward 2010; Link et al. 2019). As with many invasive shrubs (Collier et al. 2002; Flory and Clay 2010), dense barberry patches are often associated with precipitous declines (>33% for certain species) in the abundance of native flora (Kourtev et al. 1998). Silander and Klepeis (1999) attributed declines in native species to interspecific competition for light, however, they did not consider if seed and seedling predators might be partly responsible. At two of our study locations (see below), Link et al. (2018) found a dramatic reduction (82%) in native tree seedling density beneath barberry canopies. They hypothesized that these reductions may have been due to seed predators because tree seedlings transplanted under barberry canopies, thus bypassing the vulnerable seed and small seedling phase, grew at similar rates relative to those planted in nearby controls (Link et al. 2019). Williams et al. (2009) did not detect higher small mammals' capture rates beneath barberry patches but they focused on only a single target species rather than entire small mammal assemblages. Nonetheless, a few studies suggest that the presence of invasive shrubs (such as *Rhamnus cathartica* and *Lonicera maackii* in North America) promotes small mammal foraging activity (Orrock et al. 2015, Guiden and Orrock 2017, Guiden et al. 2019).

Here, we tested the hypothesis that small mammals would be more abundant beneath dense barberry patches or would forage longer in these patches or both. We quantified foraging intensity by estimating giving-up densities (GUDs), defined as the proportion of seeds consumed when mixed with an inorganic substrate (Bedoya-Perez et al. 2013). We predicted that 1) small mammal capture probability would be higher beneath dense patches of barberry, 2) experimental removal of barberry would decrease the capture of small mammals, 3) GUDs would be lower in patches that lacked a dense canopy of barberry or where barberry had been removed, and 4) our results would not vary significantly among three forest reserves separated fairly widely in space. The latter prediction goes to the issue of generality and, if true, suggests that our results are less likely to be location-specific. We used a before/after, control/impact (BACI) experimental design where patches of dense barberry were cleared midway through our field season to assess if removing an invasive regulated the foraging of vertebrate seed predators.

Methods

Plot selection

We conducted this study from May to September 2018 within closed canopy forest at three protected temperate forest reserves (hereafter referred to as locations) in southwestern Pennsylvania, USA: the Eden Hall campus of Chatham University (157 ha, 40.6638N, 79.9559W), Irwin Run Conservation Area (29.5 ha, 40.6242N, 80.0053W), located about 9.6 km from the Eden Hall campus, and Latodami Nature Center (101.1 ha, 40.6207N, 80.0297W) located about 11 km from Eden Hall (Fig. 1). The forests are within proximity to mixed-use residential zones in the suburban Pittsburgh metropolitan area. All three locations consisted of second growth deciduous forest interspersed variously with patches of coniferous forest, agricultural land, and urban land cover (Link et al. 2018). Canopy tree species at each location consisted primarily of *Quercus* spp., *Prunus serotina*, *Sassafras albidum*, *Acer rubrum*, and *Carya* spp.

Urban forests throughout this region are characterized by overbrowsing due to high densities of white-tail deer (Carson et al. 2014). Invaded and removed plots did not differ significantly in barberry density or biomass among the invaded and pre-removal plot types (Appendix 1: Fig. A1).

Experimental design

Our experiment used a before/after, control impact (BACI) design with small mammal captures and foraging behavior assessed as dependent variables in the presence versus absence of barberry. We had three plot types at all three locations: plots with a dense understory layer of barberry throughout the sampling period (hereafter invaded plots), plots where barberry had been manually removed midway through the field season with observations made before and after removal (hereafter removal plots), and nearby control plots lacking a contiguous barberry understory. We only used barberry patches containing ≥ 50% areal cover of barberry, which commonly occurred at all three study locations and elsewhere throughout much of the invasive range of barberry (Ward et al. 2009, Ward and Williams 2011). Before establishing our plots, we surveyed barberry patch boundaries by walking the perimeter of contiguous barberry patches with a GPS. Mean (± 1 standard deviation) patch size was 2009 ± 3211 m^2, with the largest patch size (9653 m^2) at the Eden Hall location and the smallest patch size (13 m^2) at the Irwin Run location (Fig. 1). Sixty total plots were distributed throughout the three locations: five of each plot type at Irwin Run and Latodami, and ten of each plot type located at Eden Hall. Plots were randomly chosen, spaced a minimum of 5 meters apart, and positioned at least 5 m from obvious habitat features that might affect mammal behavior, such as concentrations of large woody debris (Fig. 1). Invaded and removed plots were positioned approximately 5 m within the boundary of a barberry patch. Control plots were randomly selected 10–50 m away from barberry patches but lacked a dense barberry layer.

The removal plots were surveyed as invaded plots until midway through the field season when we removed barberry using a mechanical pole saw from July 16th to July 20th, 2018. A square, 25 m^2 area centered on the plot location was entirely cleared of barberry at removal plots. We did not leave trap or use seed trays for one week immediately after barberry removal then, resumed sampling as before. We quantified barberry density and stem diameter within two randomly selected positions immediately adjacent to invaded and removed plot points using a 1 m^2 grid. We used stem diameters to estimate barberry aboveground biomass allometrically using equations from Link et al. (2018).

Small mammal trapping

We used Sherman live traps (50.8 mm × 63.5 mm × 165.1 mm) to quantify capture rates (Yahner 1992, Royo and Carson 2008). On trap dates, a single trap was deployed in all 60 plots at all locations and was baited with a peanut butter/oatmeal/

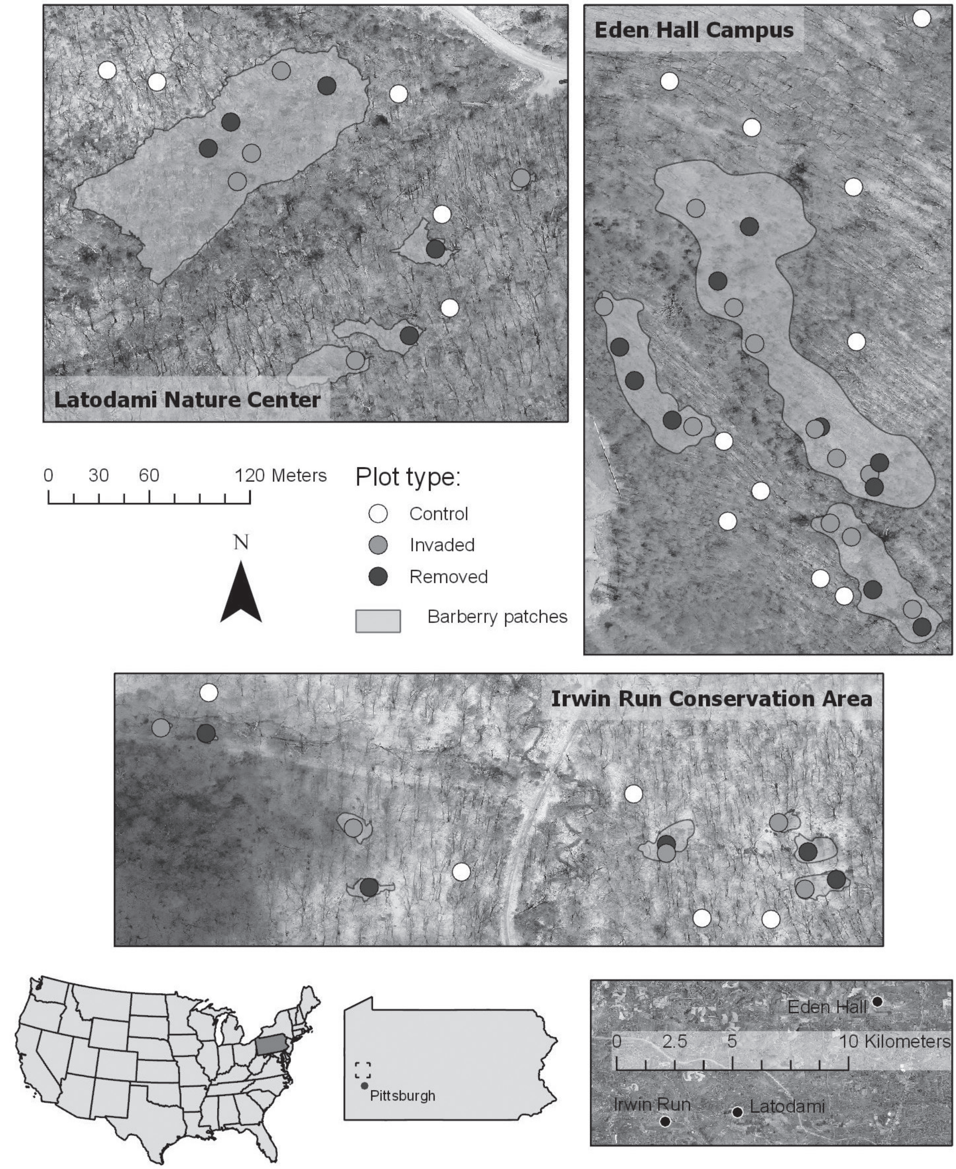

Figure 1. Map of study locations, barberry patches, and approximate positions of plots. The three detailed location maps are illustrated at the same spatial scale. Plot positions were randomly selected within 5 m of the barberry patch perimeters and away from idiosyncratic habitat features (such as downed tree crowns). Points show the approximate positions within 6 m, because the overstory canopy or topographic features or both reduced the accuracy of global positioning systems.

gelatin mixture (Yahner 1992, Royo and Carson 2008). Gelatin was used to preserve shape and consistency. Traps were set for 3–4 consecutive days every other week in June, July, and August (660 trap nights) and checked approximately every 12 hours.

We weighed and identified all captured mammals then released captive individuals, unmarked, adjacent to each plot. Small mammal capture protocols were approved by the Institutional Animal Care and Use Committee of Chatham University in accordance with the American Society of Mammalogists' guidelines for the use of wild animals (Sikes 2016). Disturbed or damaged traps were omitted from subsequent analyses. By the end of the field season, 491 evenings' worth of data were obtained across all plots and locations.

Giving up densities

We used seed buckets placed within each of the 60 plots deployed over 24-hr periods every other week throughout the field season to quantify GUDs of small mammals (Jacob and Brown 2000, Mattos and Orrock 2010). Seed bucket deployment weeks alternated with live trapping weeks to ensure that the two metrics did not conflict with each other. Seed buckets were 3.8-liter translucent round plastic containers with detachable lids (Jacob and Brown 2000; Mattos and Orrock 2010). Two 5 cm diameter holes were cut into opposite sides of the containers about 1 cm from the bottom to allow small mammal access (Mattos and Orrock 2010). Buckets were covered with a lid to prevent seed predation by birds or cervids and loss of contents from inclement weather (Mattos and Orrock 2010; Jacob and Brown 2000; Mattos and Orrock 2010). Each bucket received 6 grams of millet mixed with 200 mL of commercial play sand (Jacob and Brown 2000; Mattos and Orrock 2010). Following deployment, the millet and sand mixture was dried at 100 °C for 3 hours (to facilitate separation), the remaining millet was sieved from the sand, and weighed to evaluate foraging rate as a percentage of seed remaining (following Brown 1988). We deployed seed buckets at each location twice prior to the barberry removal dates and twice after the removal date. We removed replicate buckets that had been disturbed (15.5%) from subsequent analyses. By the end of the field season, 191 seed bucket evenings were recovered from all plots.

Statistical analysis

We assessed our findings using either linear or generalized linear mixed effect (GLMM) regression models. To avoid temporal pseudoreplication (Hurlbert 2013), our data were first divided into periods before and after the barberry removal and analyzed separately. Our dependent variables (captures and GUDs) were inherently not normally distributed. Therefore, capture probability was modeled using a GLMM with a binomial error distribution and with location as a fixed effect and plot as a random effect (to account for repeated measures). GUDs were analyzed by logit-transforming the proportion of remaining millet then averaging each GUD per plot. Averages were assessed using a linear model with location and an interaction term between location and plot type included as fixed effects. Location and an interaction term was included as a fixed effect in models to 1) account for variability in dependent variables among locations but also to 2) allow for determination of whether dependent variables varied among locations and, when that was the case, 3) if the effects of experimental treatments were consistent among locations. All statistical analyses were conducted in R version 3.5.2 (R Foundation for Statistical Computing 2019) and the GLMM model was assessed using the package *lme4* (Bates et al. 2019).

Results

We captured 23 small mammals in total: eight *Peromyscus leucopus*, ten *Peromyscus maniculatus*, four *Tamias striatus*, and a single *Blarina brevicauda*. Prior to the barberry removal treatment, capture probability significantly varied among the three study locations but not among experimental treatments (Table 1). However, during the period following barberry removal, capture probability was marginally statistically significantly different among treatments, with capture probability elevated in invaded plots relative to control and treatment plots (Table 1, Figure 2A). Before barberry removal, GUDs varied among study locations but not treatments (Table 1, Figure 2B). Following barberry removal, GUDs declined overall and did not vary among locations but values were statistically significantly greater in invaded plots relative to control and barberry removal plots (Figure 2C). No models included statistically significant terms for interactions between treatment and location.

Table 1. Results of linear and generalized linear mixed models predicting dependent variable responses to experimental treatments and plot location. Linear models correspond to F-values while generalized linear models report χ^2-values.

Model	Before or after barberry removal	Term	F- or χ^2-value $_{(df)}$	p-value
Giving- up density linear model, logit-transformed	Before	Location	$7.7_{(2,50)}$	0.0012
		Treatment	$1.0_{(2,50)}$	0.3425
		Interaction	$0.6_{(4,50)}$	0.6894
	After	Location	$0.2_{(2,50)}$	0.7922
		Treatment	$3.9_{(2,50)}$	0.0259
		Interaction	$0.5_{(4,50)}$	0.7004
Trap rate generalized linear mixed model	Before	Location	$0.3_{(2)}$	0.3014
		Treatment	$2.4_{(2)}$	0.8391
		Interaction	$0.2_{(2)}$	0.9933
	After	Location	$0.1_{(2)}$	0.9817
		Treatment	$4.7_{(2)}$	0.0946
		Interaction	$0.1_{(2)}$	0.9988

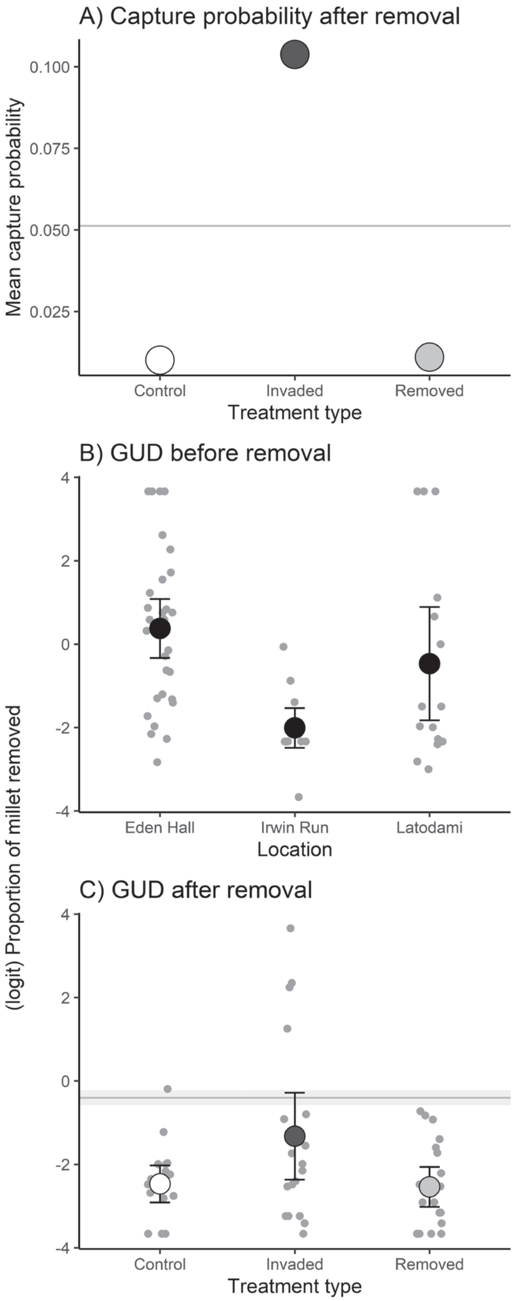

Figure 2. A Mean capture rates of small mammals during the period after experimental barberry removal delineated by plot type. The horizontal line illustrates the mean capture rate across all locations and treatments during the period prior to experimental barberry removal **B** mean (± 95% confidence intervals) giving-up densities delineated by location before the experimental barberry removal and **C** by treatment type for the period after barberry removal. The line and associated gray ribbon illustrate the mean ± 95% confidence interval for giving-up densities across all treatments and locations before barberry removal.

Discussion

Results affirmed our hypotheses and provide evidence that small mammals forage more aggressively under invasive shrub canopies. During the latter half of the field season, both the small mammal capture probability and GUDs were significantly higher in plots with recalcitrant barberry layers relative to both control plots and plots where barberry was removed. Differences in capture probability and GUDs were not detected in the first half of the field season and we observed a decrease in small mammal GUDs between early and late summer. However, comparable seasonal patterns in activity have been observed for at least *P. leucopus* in similar ecological settings elsewhere (McMurry et al. 1996). Although we cannot rule out the possibility that the activity of removing barberry itself altered the behavior activity of small mammals after the event, dependent variables in control plots lacking barberry were nearly identical to barberry-removed plots during the second half of the field season. Therefore, we conclude that small mammal foraging activity under barberry canopies is elevated relative to forest floors lacking the invasive species.

Dense patches of a suite of invasive shrub species are now widespread throughout much of the Eastern Deciduous Forest Biome of North American and often cause major declines in native plant diversity (see introduction). Research exploring the mechanisms behind these patterns to date has typically attributed declines in diversity to interspecific resource competition between nonnative shrubs and native species, biological or chemical changes to soil environments that occur after invasion, or invasive species-driven allelopathic effects (Hierro and Calloway 2003; Morales and Traveset 2009; Skurski et al. 2014). Our findings, and those of several others (Mattos and Orrock 2010; Dutra et al. 2011; Malo et al. 2013; Guiden and Orrock 2019) provide an alternative, though not mutually exclusive, explanation. Specifically, seed and seedling predation reduces flora diversity because small mammals occur more frequently and forage longer beneath dense shrub patches, thereby promulgating apparent competition (Holt 2017) between invasive and native flora. The phenomenon is not limited to nonnative taxa: indeed, dense understories of native shrubs and ferns also increase the activity of small mammals and levels of seed predation (Yahner 1986; Carey and Harrington 2001; Castleberry et al. 2002; Spencer et al. 2005; Royo and Carson 2008).

Nonetheless, nonnative shrubs may be more likely to create dense understory layers than native plant species because herbivores typically avoid them (Grosholz 2010; Averill et al. 2016). In our case, barberry may provide particularly effective cover from enemies because it has thorns and it grows via multiple stems that radiate densely from a central root crown (Gleason and Cronquist 1991; Ehrenfeld 1999). Overall, our results, combined with those of others, suggest that the spread of numerous nonnative shrubs across eastern forests, may increase predation pressure on vulnerable seeds and seedling across broad regions wherever these shrubs occur and are abundant. Because of ubiquity of invasive woody plants globally (Royo and Carson 2006; Richardson and Rejmánek 2011), our findings could apply beyond the eastern U.S., and call for replicated experiments in invaded areas worldwide.

Other mechanisms whereby nonnative shrubs increase the abundance or foraging activity of small mammal activity exist beyond providing dense cover. Invasive species may augment small mammal populations if they produce edible fruits and seeds (Orrock et al. 2015). In our case, barberry produces a fruit that is palatable to birds and mammals (Silander and Klepeis 1999; Hayes and Holzmueller 2012) and thus may be augmenting food resources. However, dense patches of invasive shrubs may not always enhance the abundance and foraging activity of small mammals (Hayes and Holzmueller 2012). For example, nonnative shrubs can reduce the abundance of native plant species that are key food resources, or the nonnatives displace native woody vegetation that provided better cover than the nonnative shrubs (e.g., large seeds; Lambrinos 2000; Ostoja et al. 2009; Freeman et al. 2014; Lucero and Callaway 2018). Overall, dense patches of nonnative shrubs can increase small mammal abundance or concentrate foraging because they provide dense cover, the cover extends earlier or later during the growing season, or the nonnatives augment food resources.

Prior investigations in our study locations have detected evidence that seed or seedling predation causes the low densities of native tree seedlings beneath barberry understories. Specifically, Link et al. (2018, 2019) bypassed the seed germination and early establishment phase by transplanting tree seedlings (> 20 cm tall) under barberry and within adjacent control areas lacking barberry. No differences in the survival or growth rates of *Quercus* spp. and *Prunus serotina* seedlings between barberry patches and control patches lacking barberry were detected, suggesting that vertebrate seed and seedling predators kill seeds or young seedlings, whereas larger individuals are less vulnerable, though alternative explanations cannot be ruled out (e.g., allelopathy). Still, Guiden and Orrock (2017) and Bartowitz and Orrock (2016) found that an invasive shrub (*Rhamnus cathartica*) provided cover for small mammals, which increased rates of seed predation rates from 60 to nearly 100%. Furthermore, if the seeds or foliage of dominant invasive species are unpalatable, this may increase rates of seed predation and herbivory on any remaining native plant species growing nearby (Orrock et al. 2008; Dangremond et al. 2010). Regardless, vertebrate seed and seedling predation may be a common mechanism underlying some of the declines in native plant species diversity, particularly woody species with larger seeds (Bartowitz and Orrock 2016).

Seasonal changes in population densities, resource availability, and perceived cover from predation may mitigate how recalcitrant invasive plant cover affects small mammals. Such findings suggest that, during certain seasons, the effects of an invasive plant on foraging activity may diminish. Barberry leaves were present during our entire study period. However, nonnative shrubs may leaf out earlier in the spring and senesce later in the fall than native species, thus extending the phenological window whereby these nonnatives provide small mammals a refuge. Nocturnal light intensity (Prugh and Golden 2014; Guiden and Orrock 2019) and the relative abundance of palatable seeds from canopy trees (Schnurr et al. 2002), neither of which were controlled for in this study, may also significantly impact small mammal foraging behavior. Predation risk may also vary with perceived food quality, as higher-quality resources can result in greater risk-taking (Utz et al. 2016). Therefore, the effects of recalcitrant invasive species cover on small mammals may not be as strong during evenings with low moon luminescence, especially during heavy mast years.

Conclusion

Our findings highlight the potential for elevated foraging intensity in a key faunal assemblage resulting from invasive plants that form recalcitrant understories. Prior studies (Link et al. 2018, 2019) conducted in our field locations revealed depressed tree seedling densities under barberry but transplanted seedlings planted under barberry survived and grew at rates similar to control subjects. Such results, in combination with those presented here, suggest that small mammal foraging may be limiting recruitment of native trees or other heavily foraged plant taxa. Thick understory growth of *R. cathartica*, another invasive shrub spreading through temperature deciduous forests of North America, also elevates small mammal foraging rates and reduces the abundance of foraged native plants as a result (Guiden and Orrock 2017). However, very few studies have addressed such hypotheses despite the large number of invasive shrubs currently spreading through forest understories in North America and elsewhere.

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Appendix 1

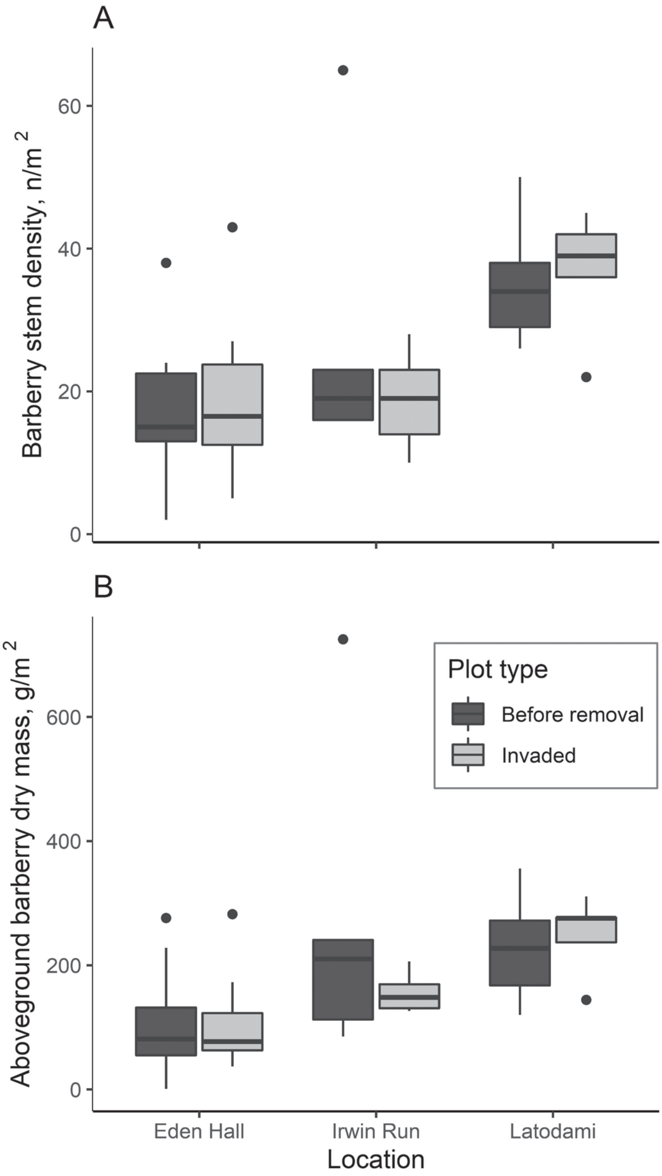

Figure A1. Distribution of **A** barberry stem densities and **B** barberry aboveground biomass in removal (prior to eradication) and invaded plot types, delineated by location. No barberry plants were recorded in control plots. Stem densities were assessed with a generalized linear model and Poisson error distribution; values significantly varied among locations ($\chi^2 = 87.4$, df = 2, $p < 0.0001$) but not plot types ($\chi^2 = 0.7$, df = 1, $p = 0.4175$). Barberry biomass was assessed with a linear model; values significantly varied among locations ($F_{2,36} = 5.7$, $p = 0.0073$) but not plot type ($F_{1,36} = 0.5$, $p = 0.4891$).

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RESEARCH ARTICLE

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Clonal growth strategies of *Reynoutria japonica* in response to light, shade, and mowing, and perspectives for management

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Abstract

Many of the most invasive plant species in the world can propagate clonally, suggesting clonality offers advantages that facilitate invasion. Gaining insights into the clonal growth dynamics of invasive plants should thus improve understanding of the mechanisms of their dominance, resilience and expansion. Belonging to the shortlist of the most problematic terrestrial invaders, *Reynoutria japonica* var. *japonica* Houtt. (Japanese knotweed) has colonized all five continents, likely facilitated by its impressive ability to propagate vegetatively. However, its clonal growth patterns are surprisingly understudied; we still do not know how individuals respond to key environmental conditions, including light availability and disturbance. To contribute to filling this knowledge gap, we designed a mesocosm experiment to observe the morphological variation in *R. japonica* growth in homogeneous or heterogeneous conditions of light stress (shade) and disturbance (mowing). Rhizome fragments were planted in the middle of large pots between two habitat patches that consisted of either one or a combination of the following three environmental conditions: full light without mowing, full light with frequent mowing, or shade without mowing. At the end of the experiment, biomass and traits related to clonal growth (spacer and rhizome lengths, number of rhizome branches, and number of ramets) were measured. After 14 months, all individuals had survived, even those frequently mowed or growing under heavy shade. We showed that *R. japonica* adopts a 'phalanx' growth form when growing in full light and a 'guerrilla' form when entirely shaded. The former is characteristic of a space-occupancy strategy while the latter is more associated with a foraging strategy. In heterogeneous conditions, we also showed that clones seemed to invest preferentially more in favorable habitat patches rather than in unfavorable ones (mowed or shaded), possibly exhibiting an escape strategy.

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These observations could improve the management of this species, specifically by illustrating how aggressive early control measures must be, by highlighting the importance of repeated mowing of entire stands, as this plant appears to compensate readily to partial mowing, and by informing on its potential responses towards the restoration of a cover of competitive native plants.

Keywords

clonal growth, environmental heterogeneity, *Fallopia japonica* (*Polygonum cuspidatum*, Japanese knotweed), invasion dynamics, lateral expansion of patches/stands, spatial spread, vegetative regeneration

Introduction

Clonality is an attribute frequently associated with plant invasiveness (Lloret et al. 2005), and many of the most invasive plants in the world are clonal (Liu et al. 2016; Lowe et al. 2000; Pyšek and Richardson 2007). This is not surprising, as the highly plastic modular growth form of clonal plants releases them from many constraints related to being sessile. In clonal plants, resource-acquiring structures (leaves and root tips) are located on units called ramets (i.e. potentially autonomous physiological individuals; in other words, aerial shoots and their associated roots) that are projected into the environment by stolons or rhizomes, which serve as spacers between other resource-acquiring structures (Hutchings and de Kroon 1994). Although ramets are fixed in space, whole clonal fragments (i.e. physical individuals composed of all interconnected ramets) can spread laterally and may exhibit a large mobility (Oborny and Cain 1997; Zobel et al. 2010). By plastically changing the length, direction and/or number of spacers, clonal plants are able to exhibit complex behaviors such as precision foraging and selective ramet placement, escape strategies, or division of labor through ramet specialization (de Kroon and Hutchings 1995; Gao et al. 2012; Hutchings and Wijesinghe 1997; Oborny et al. 2012). These highly adaptable abilities can even potentially be transferred asexually through epigenetic trans-generational inheritance (Latzel and Klimešová 2010; Latzel et al. 2016). Clonality also enables survival and persistence of populations in absence of sexual reproduction (Eriksson 1997), rapid cover of and dominance of invaded sites (Herben and Hara 1997; Pyšek 1997) and, through clonal integration, the exchange of water, nutrients, carbon and information between connected ramets to reduce resource shortages and mitigate the effects of stress and disturbance (Jónsdóttir and Watson 1997; Liu et al. 2016). Out of these many advantages, some have been shown to be particularly associated with invasiveness such as high root foraging abilities or clonal integration (Keser et al. 2014; Song et al. 2013). Yet, many unknowns remain regarding the link between clonality and invasiveness, and the study of clonal invaders can help lay the foundation required to address fundamental and applied ecological questions (Liu et al. 2016; Yu et al. 2016).

At the local scale, since performance and impact of invasive clonal plants are often directly related to their clonal growth characteristics (e.g. architectural traits, lateral growth rate, ramet density, clonal integration, growth strategies), understanding clonal growth patterns and strategies is of prime importance for improving management strategies. This is why the clonal growth dynamics of many highly problematic clonal invaders have been the subject of extensive research over the years: e.g. *Phragmites australis* (Amsberry et al. 2000; Bellavance and Brisson 2010; Douhovnikoff and Hazelton 2014), *Carpobrotus edulis* (Roiloa et al. 2010; Roiloa et al. 2013), *Solidago* spp. (Hartnett and Bazzaz 1983; Jakobs et al. 2004; Stoll et al. 1998).

Despite being listed as one of the worst invasive plants in the world (Lowe et al. 2000), Japanese knotweed (*Reynoutria japonica* var. *japonica* Houttuyn) and its strong clonal growth abilities remain understudied. Capable of early and rapid growth by remobilizing resources stored in its rhizomes, *R. japonica* often forms dense monoclonal stands that exclude many native species and are also a nuisance for various human activities (Beerling et al. 1994; Lavoie 2017). Populations of *R. japonica* are extremely difficult to control (Child and Wade 2000; Delbart et al. 2012) and have, mainly by vegetative spread, successfully colonized most temperate regions of the world (Alberternst and Böhmer 2006; Beerling et al. 1994). In its native range, in the specific environment of Japan's high elevation volcanic deserts, several studies reported that clonal fragments of *R. japonica* var. *compacta* expand by reiterating a fixed pattern of sympodial rhizome growth (at the end of which clumped ramets are produced) with a high degree of clonal integration among ramets of different size to avoid asymmetric competition (Adachi et al. 1996a; b; Suzuki 1994). In its introduced range however, investigations on the clonality of *R. japonica* have mainly focused on its regeneration capacities (e.g. Bímová et al. 2003), or on resource translocation (Price et al. 2002). Two modelling studies also tried to understand the development of *R. japonica*'s clonal fragments by implementing growth rules derived from the Japanese studies. Yet, they recognized that their results were subject to serious restrictions due to the lack of quantitative data on the variability of clones' growth and demography in various environmental conditions (Dauer and Jongejans 2013; Smith et al. 2007). Consequently, we are still ignorant of how clonal fragments of *R. japonica* grow and expand in the wild and how they respond to environmental factors.

Two important features of environments that correspond to two main means of managing *R. japonica* are light availability and disturbance. *Reynoutria japonica* is mainly found in high-light habitats, but closed-canopy habitats such as forests can still be colonized either directly from vegetative propagules, or from the lateral expansion of surrounding populations (Beerling et al. 1994; Tiébré et al. 2008). Because closed-canopy habitats diminish the performance of *R. japonica*, restoration using competitive native species is increasingly used in control efforts (Dommanget et al. 2013). While many types of disturbance may facilitate invasion of *R. japonica* (Martin 2019; Pyšek and Hulme 2005), mowing is a disturbance that is used as the main control technique against *R. japonica* in many regions. Mowing is done either across entire stands or just on part of a stand, for example on roadsides or at the border between two properties (Delbart et al. 2012; Schiffleithner and Essl 2016). It is likely that the clonal dynamics of *R. japonica* vary substantially between the possible combinations of these environmental factors: i.e. whole connected clones or parts of clones growing in full light or under a closed canopy, and being mowed or not. For instance, some authors report that shaded clones usually display a lower ramet density than the ones growing in open areas (Dommanget et al. 2019; Martin et al. 2019) or that, conversely, mowing increases ramet density and favors stands' expansion (Beerling 1990; Child and Wade 2000).

To improve our understanding of *R. japonica*'s invasion dynamics, specifically how clonal growth responds to important environmental factors, we designed a mesocosm experiment. In it, we explore how the development and expansion of young clonal fragments is affected by homogeneous or heterogeneous conditions of light stress (shade) and disturbance (mowing). We aimed to better understand plant growth strategies and potential trade-offs when faced with more or less favorable habitats, and investigate how these responses might be relevant to improved management of *R. japonica* by mowing/cutting or by ecological restoration using dense cover of competitive species. We hypothesized that: i) a homogeneously high light availability would favor aggregation of ramets while a homogeneous shade would favor a more scattered distribution of aerial shoots, two growth forms respectively known as phalanx and guerrilla (sensu Lovett Doust 1981); ii) mowing would release axillary buds from apical dominance, therefore favoring higher rhizome branching frequency and ramet density than in un-disturbed *phalanx* clones; and iii) individuals that are partially stressed (shaded) or disturbed (mowed) would try to 'escape' these less favorable habitats by investing more in the growth of their parts growing in high light and undisturbed areas.

Materials and methods

Biological material

In April 2017, rhizomes belonging to a single *R. japonica* individual were manually excavated. The plant was located outside the village of Cholonge (1061 m a.s.l.; 45°00'N–5°79'E), in the French Alps. This individual was chosen because it was growing in an open and unmanaged site. This was an important prerequisite since we wanted to limit the chance that ramets growing from its rhizome fragments were influenced by stressful or disturbed conditions via transgenerational inheritance (Latzel and Klimešová 2010; Latzel et al. 2016). Recent evidence showed that a single *R. japonica* genotype can exhibit differing phenotypes determined by the growing conditions of their "parental" populations, due to epigenetic variation (Zhang et al. 2017).

Following excavation, rhizomes were washed and cut to obtain homogenized fragments with the same approximate weight and number of nodes. The thirty most similar fragments were selected, bagged and stored in a cold room before the start of the experiment. These fragments had a mean weight of 16.44 g (± 0.85 g) and a mean number of nodes of 8.06 (± 2.46).

Experimental design

The mesocosm experiment was conducted in an experimental nursery of the National Forest Office (ONF) located in Guémené-Penfao, Brittany (France). The area is characterized by mean monthly temperatures ranging from 7.9 to 16.4°C, and 694 mm of mean annual precipitation (data from Rennes meteorological station; www.meteofrance.com).

The experimental design was composed of five treatments with six replicates each. The treatments were designed to enable us to evaluate how *R. japonica* responds to homogeneous or heterogeneous environmental stressors. Each plant was grown in pots divided into two habitat patches. These habitat patches were identical for homogeneous treatments: light without mowing (L), light with mowing (M), and shade without mowing (S). For heterogeneous treatments, they differed: half-light – half-mowing (LM) and half-light – half-shade (LS); Fig. 1).

Large pots for this experiment were created from thirty rainwater tanks of ca. 1000L (120 × 100 × 116 cm) by cutting off their tops. Pots were first filled with a 15 cm layer of gravels (Ø 0–32 mm) to facilitate water drainage through an outlet pipe. On top of

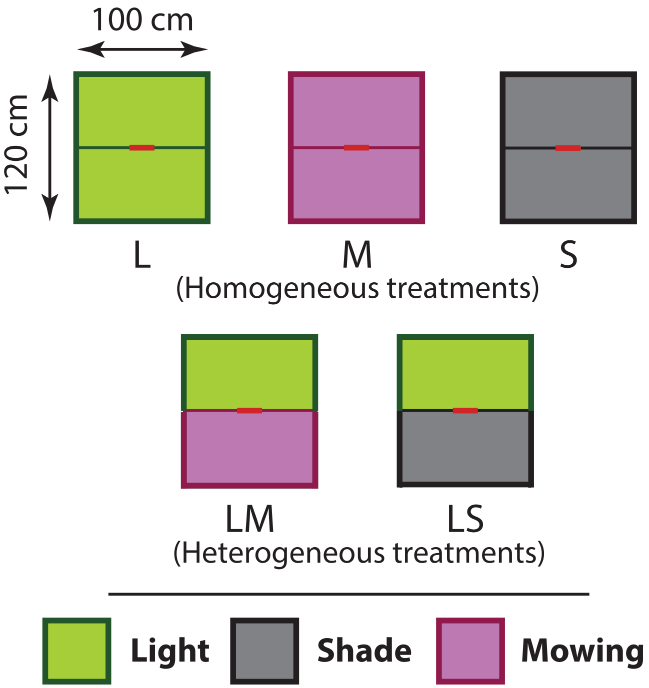

Figure 1. Experimental design. The different colors represent the treatments: green (un-shaded and un-mowed habitat), pink (un-shaded but mowed habitat) and grey (shaded but un-mowed habitat). Each of these five different treatments had six replicates. The red segments in the middle of "pots" represent the position of the rhizome fragments that were planted

that, we added approximately 100 cm layer of a certified substrate composed of 70% river sand, 15% loam and 15% compost (chemical composition of the substrate: N = 1.2%; K_2O = 1.4%; P_2O_5 = 0.4%; MgO = 0.6%; CaO = 2.1%: C:N = 12; pH = 8–9). Shade treatments (S and LS) were created by inserting 3 m poles into the pots and covering them with netting that filtered around 80% of the light. Pots were arranged in a flat area, with their location and orientation randomly chosen in such a way that each replicate of a given treatment had a different orientation from the other five replicates. To avoid the effect of projected shadows caused by the tall shade treatments, pots were separated by 4 m intervals in every direction. Additionally, the randomized placement and orientation of all the pots was reshuffled in the middle of the experiment.

In early May 2017, the thirty rhizome fragments were randomly assigned to one of the pots. They were buried two centimeters below the surface in the middle of the pots, orthogonally to the greater length of the pots. This position coincided with the limit between the two habitat patches of the pots (Fig. 1). In mowed habitats (M and LM), the aerial shoots of ramets were manually clipped and removed every time they reached approximately 25 cm in height. This resulted in three mowing events during the first vegetative season, and one during the following spring two weeks before the end of the experiment. This mowing frequency was chosen because it reflects regular management along many French roads and railways. Throughout the experiment, pots were weeded regularly and water availability was maintained using a multi-point dripping irrigation system. We also checked for potential differences in air temperature and soil humidity using ten TMS-4 data-loggers (www.tomst.com) randomly placed in treatments' modalities, left two weeks, and moved to another random replicate of the same modality.

Harvest and measurements

Before each mowing event, the number of ramets in each habitat patch was recorded. We decided to stop the experiment when ramets began to reach pots' edges, to minimize obstacle-effects on the clonal architecture of the plants (duration of the experiment = 420 days; ca. 14 months).

The experiment was harvested at the end of June 2018. All ramets growing aboveground in all habitat patches were counted before being clipped and oven-dried for 48h at 100 °C prior to measuring dry biomass. Additionally, the horizontal distance between the farthest ramet and the center of the pot (i.e. the location of the rhizome fragment initially planted) was measured for each half of each pot to estimate maximal lateral expansion distances for developing stands. We then carefully excavated the plants, using mostly our hands and screwdrivers in order not to break fragile rhizomes and buds, and to extract rhizomatous systems as intact as possible. However, roots were intentionally cut to facilitate excavation, as our hypotheses were unrelated to the root system. We then marked the position of the separation line between the two habitat patches on each rhizomatous system before removing the dirt with an air compressor and brushes. Rhizome and spacer lengths (see below), number of rhizome branches, and number of axillary and basal buds that were growing in each habitat patch were measured. Finally, we also measured rhizome biomass with the same method as for aboveground tissue.

We follow Sachs' (2002) definition of "spacers" as plagiotropic stems, in this case rhizomes, whose apices produce new ramets. Rhizomes, however, do not always produce ramets as they may be dormant, broken, dying, or still growing. Consequently, all *R. japonica* spacers are rhizomes (with new ramets at their end), but not all rhizomes are spacers. In this study, spacer length corresponds to the distance between the location on the mother rhizome whence the spacer branched and the first aboveground node of the nascent ramet's aerial shoot.

Statistical analyses

Prior to analyses, data were explored and prepared following the protocol of Zuur et al. (2010). When required, data were log transformed to ensure homoscedasticity among groups.

Since *R. japonica*'s clonal growth patterns and processes are largely unknown, the first steps of our analyses were necessarily exploratory and descriptive. To investigate our hypotheses however, the responses of variables characterizing *R. japonica*'s growth form and strategies were analyzed more thoroughly. These variables were biomass (aboveground, rhizomatous and total dry biomasses in grams), specific spacer length (length of a spacer per unit of biomass), number of ramets (accounted for as the number of aerial shoots) and rhizomes' branching frequency (calculated as the number of rhizome branches per unit of rhizome length). Analyses were performed at two different scales: (i) pots and (ii) half-pots. As a reminder, in our experimental design there were two habitat patches per pot, identical or not, but only one plant (Fig. 1), that is, one *R. japonica* individual/clonal fragment. Consequently:

(i) At the *pot scale*, measurements made within the two habitat patches of each pot were summed up so as to have observations at the individual level. As such, whole plants were taken as statistical units and our five treatments (L, M, S, LM, and LS) were used as explanatory factors. For each response variable, we performed ANCOVAs with type II Sums of Squares and used the weight and number of nodes of initially planted rhizomes as covariates. For multiple comparisons, we used pairwise *t*-tests using Holm-Bonferroni corrections to control for family-wise error rates.

(ii) At the *half-pot scale*, observations were made at the sub-individual level (i.e. half plants) and differences linked to differing growing conditions between habitat patches were investigated, but only for replicates belonging to heterogeneous treatments (LM and LS). As the two half-plants of each pot were not independent, we used mixed-ANCOVAs with *pot* as a random effect (Rutherford 2011). Post-hoc tests were performed using Tukey's HSD test to account for possible violations of sphericity.

Initially, we also wanted to study potential differences in the number of buds between treatments as evidence of habitat selection, but we observed during harvest that *R. japonica* produces a bud at each node regardless of the treatment, precluding further analysis.

All analyses were performed with R version 3.5.2 (R Development Core Team 2019).

Results

General observations and biomass production

Consistent with what is reported in the literature (Adachi et al. 1996a; Bailey et al. 2009), clonal fragments expanded through the repeated sympodial branching and growth of rhizomes. One or several rhizomes emerged from the lateral buds or node meristems of the initially planted rhizomes. These new rhizomes quickly curved upward to produce ramets, and then new lateral rhizomes sprouted and grew farther off in a concave fashion to produce new ramets and so on. Unfortunately, the precise timing of rhizome branching could not be investigated with our experimental setting nor could the factors inducing branching or the upward bending of rhizomes. In all treatments, mortality of individual ramets (not whole clones) was observed during the first growing season, even in undisturbed pots, and this mortality did not seem to be related to overcrowding and intra-plant competition. Conversely, some habitat patches harbored no ramets for months until their number quickly increased after a first ramet colonization.

All rhizomes that were planted at the beginning of the experiment gave birth to clonal fragments that survived throughout the 14 months of the experimentation. Interestingly, most clones produced flowers in the first growing season except those of the entirely mowed treatment (M).

As expected, most traits related to clonal growth varied strongly by treatment (Tables 1, 2; Figs 2–5). In little more than a year, the largest clones produced over 1.9 kg of dry biomass (without roots) and over 21 m of rhizomes, with some spacers reaching 112 cm (Table 1).

Clones in the L treatment (full light without mowing) produced their farthest ramets (from their center) farther than individuals of any other treatments and with a far lower variability (Table 2). As such, after 14 months, clones growing in full light without mowing possessed a mean radius exceeding 56 cm while entirely mowed (M) or shaded (S) clones displayed mean radiuses of around 12 and 34 cm, respectively (Table 2).

In all our analyses, covariates did not significantly influence examined responses. Consequently, observed differences could be attributed to treatments. Unsurprisingly, shade and mowing treatments significantly reduced total biomass production (F = 89.36; dfn = 4, dfd = 26; $p < 0.001$), aboveground biomass production (F = 43.18; dfn = 4, dfd = 26; $p < 0.001$) and rhizomatous biomass production (F = 57.03; dfn = 4, dfd = 26; $p < 0.001$; Fig. 2a). With the exception of the pairs L-LM (t = 1.532, p = 0.145) and LM-LS (t = 1.383, p = 0.096), all differences in mean total biomass were significant. For homogeneous treatments, the hierarchy of differences remained the same for

Table 1. Summary statistics of descriptive variables measured across all treatments (at the pot scale).

	Maximum spacer length (cm)	Mean spacer length (cm)	Cumulated rhizome length (cm)	Mean rhizome length (cm)	Longest length between opposite ramets (cm)	Number of rhizome branches	Number of buds (on rhizomes)	Number of ramets (aerial shoots)	Aboveground dry biomass (g)	Rhizomatous dry biomass (g)	Total dry biomass (g)
Mean	56,07	30,59	599,2	16,65	68,98	24,6	230,42	23,97	499,45	207,04	706,49
Standard deviation	29,22	24,55	519,03	6,94	39,43	24,4	159,3	13,25	404,48	200,8	591,5
Median	53,25	23,27	484,7	16,32	68	17,5	220,5	22,5	325,5	98,38	394,52
Minimum	17,1	1	38,9	3,367	6,5	1	19	5	1,21	9,01	10,22
Maximum	112	92,5	2113	29,18	133	97	638,92	55	1221	708,66	1902,66

Table 2. Descriptive statistics by treatment for the distance to the farthest ramet in each habitat patch (pot-half).

	L	M	S	LM	LS
Mean	56,17	11,63	34,50	53,83	36,58
Standard deviation	6,70	7,06	23,85	19,31	23,46
Median	56	11,5	38	57,5	34
Minimum	45	1,5	5	15	5
Maximum	66	25	70	75	66

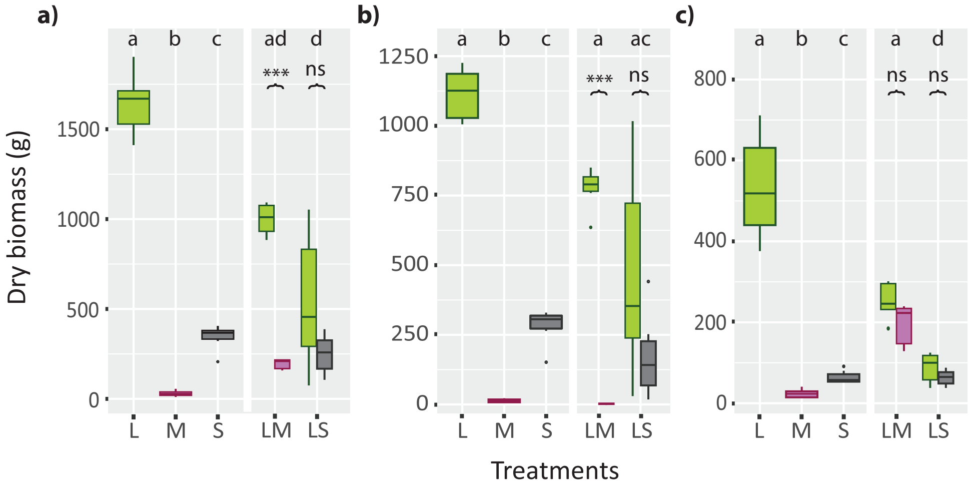

Figure 2. Differences in total dry biomass (**a**), aboveground biomass (**b**) and rhizomatous biomass (**c**) between the L (light), M (mowed), S (shaded), LM (half-light – half-mowed) and LS (half-light – half-shaded) treatments. For analyses at the pot scale, letters are used to indicate the significance level of differences (treatments not sharing the same letter were significantly different at $p < 0.05$). For analyses at the half-pot scale, stars are used to indicate significant differences between habitat patches (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; ns = not significant). As a reminder, differences among pot-halves have only been investigated for heterogeneous treatments (i.e. LM and LS).

aboveground and rhizomatous biomasses. On the other hand, although clones growing in the LM treatment did not differ significantly from the LS treatment with respect to aboveground biomass (t = -1.523, p = 0.555), they produced significantly more rhizomatous biomass (t = -3.844, p = 0.007; Fig. 2b, c). Among heterogeneous treatments, at the half-pot scale, plant parts growing in the favorable habitat patches (i.e. un-mowed and undisturbed) of the LM treatment presented a significantly higher aboveground (t = 14.609, $p < 0.001$) and total biomass (t = 12.72, $p < 0.001$) than parts growing in the mowed halves of these pots (Fig. 2a, b). No significant differences in any type of biomass production were found among habitat patches of the LS treatment, nor in rhizomatous biomass production between pot-halves of the LM treatment (Fig. 2a–c).

Spatial exploration and clonal traits

At the scale of pots, individuals of *R. japonica* growing in full light without mowing (L) had significantly lower specific spacer lengths than clones growing in fully shaded habitats (S; t = 4.361, $p < 0.001$) and entirely mowed individuals (M; t = 3.005, $p <$ 0.025). At the half-pot scale, despite a slight trend of increased specific spacer length for spacers growing in the shaded habitat patches of the LS treatment, no significant differences were found within or among heterogeneous treatments (Fig. 3).

Shading (S) led to the production of fewer ramets than full light (L) (t = -7.327, $p < 0.001$) and mowing (M) (t = -8.23, $p < 0.001$), and there was no differences

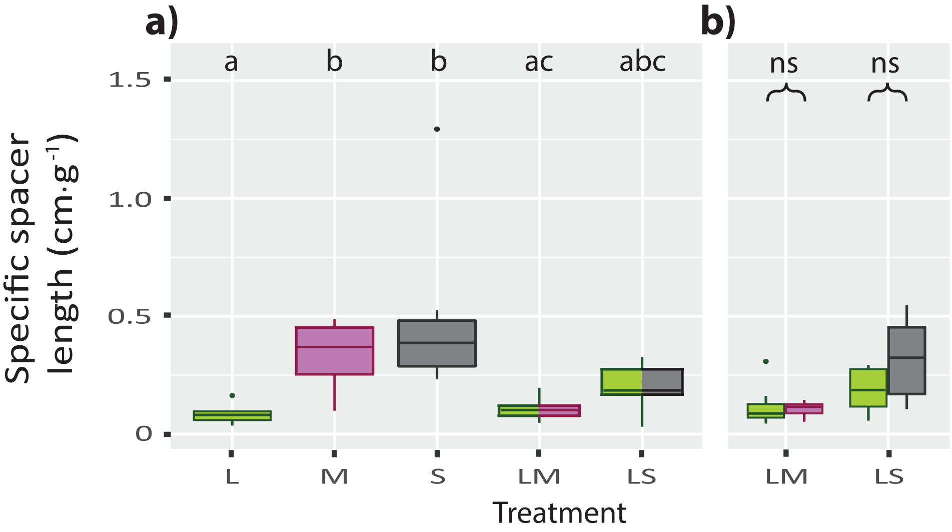

Figure 3. Differences in specific spacer length between L (light), M (mowed), S (shaded), LM (half-light – half-mowed) and LS (half-light – half-shaded) treatments at the scale of pots (**a**) or half-pots/habitat patches (**b**). Treatments not sharing the same letter are significantly different at $p < 0.05$, ns = not significant.

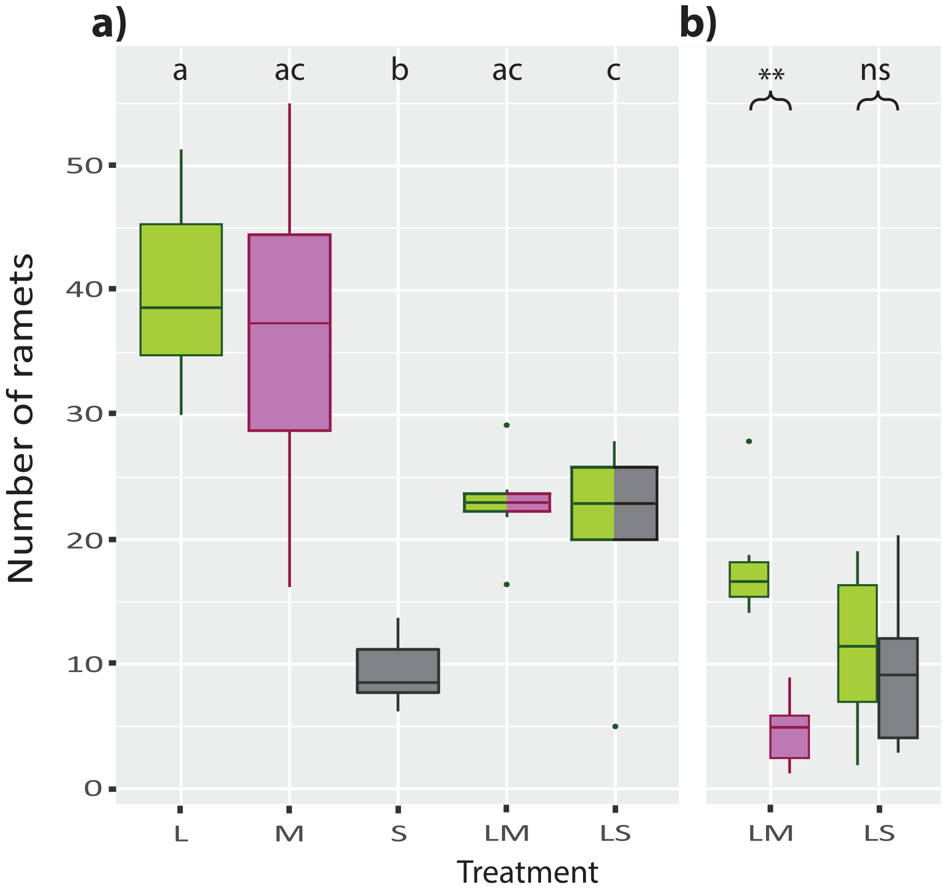

Figure 4. Differences in number of ramets between L (light), M (mowed), S (shaded), LM (half-light – half-mowed) and LS (half-light – half-shaded) treatments at the scale of pots (**a**) or half-pots/habitat patches (**b**). For the former, treatments not sharing the same letter were significantly different at $p < 0.05$, while for the latter: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; ns = not significant.

between full light and mowing in number of ramets ($t = 0.276$, $p = 0.89$; Fig. 4a). Interestingly, at the half-pot scale, the un-mowed habitat patches of the LM treatment displayed significantly more ramets than mowed habitat patches ($t = 5.73$, $p = 0.002$). Such pattern among the two habitat patches of LM treatment's individuals is therefore not consistent with the pattern observed in their homogeneous treatment counterparts (namely clones in L and M treatments) that possessed similar number of ramets (Fig. 4a, b).

Finally, clones in pots receiving full light (L) had a significantly higher rhizome branching frequency than shaded clones (S) ($t = -2.686$, $p = 0.032$), but not higher than entirely mowed clones (M; $t = 0.393$, $p = 0.267$) while LM and LS clones showed intermediate values (Fig. 5a). At the half-pot scale, once again, no significant differenc-

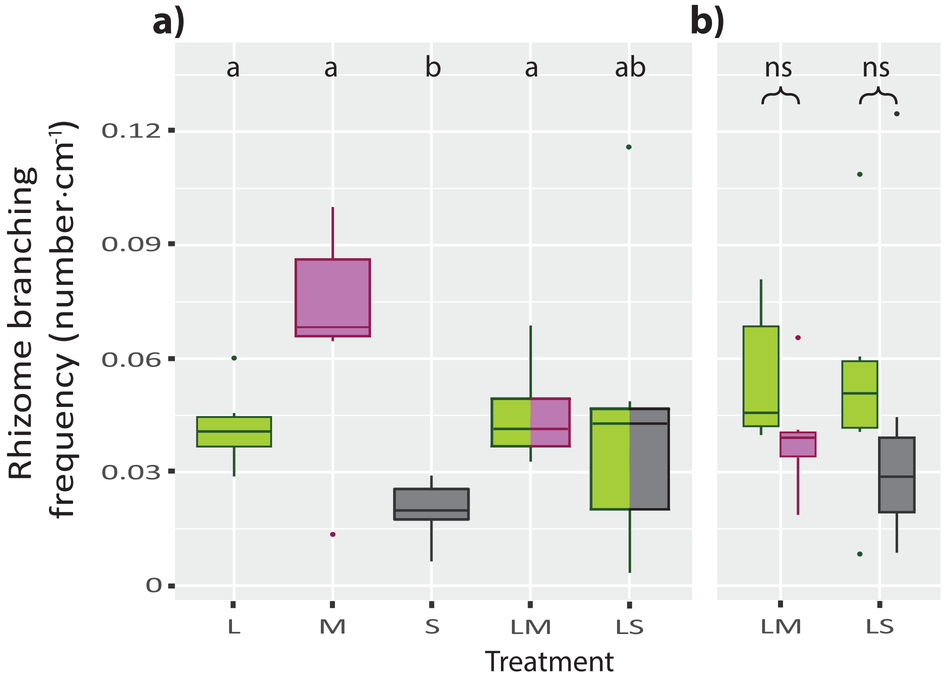

Figure 5. Differences in rhizome branching frequency (measured as the number of branches per unit of rhizome length) between L (light), M (mowed), S (shaded), LM (half-light – half-mowed) and LS (half-light – half-shaded) treatments at the scale of pots (**a**) or half-pots/habitat patches (**b**). For the former, treatments not sharing the same letter were significantly different at $p < 0.05$, while for the latter: ns = not significant.

es in branching frequency were found between plant parts growing in habitat patches of the heterogeneous treatments LM and LS (Fig. 5b). Interestingly, rhizomes in unmowed halves of LM treatments had a seemingly higher branching frequency than rhizomes in mowed ones, therefore exhibiting a reverse pattern compared to individuals growing in homogeneous treatments L and M.

Discussion

Despite its importance for understanding and managing local invasion dynamics of *R. japonica* and its congeners, the clonal growth of this taxon and its variations under various environmental conditions have been surprisingly understudied (Bashtanova et al. 2009; Smith et al. 2007). The observations and data presented here represent, to the best of our knowledge, the first quantitative assessment of the clonal growth dynamics of *R. japonica* in various homogeneous or heterogeneous habitats.

Clonal growth forms and strategies

Our results show that *R. japonica* can respond plastically to the quality of its habitat in various vegetative growth traits. In accordance with our first hypothesis, *R. japonica* adopted a phalanx growth form when growing in a homogeneously illuminated habitat by aggregating many ramets separated by short spacers. Conversely, when growing under heavy shade, clones only presented a few ramets separated by long spacers, typical of a guerrilla growth form (Figs 3a, 4a). As these two growth forms were associated with different specific spacer lengths and rhizome branching frequencies (Figs 3a, 5a), they likely stemmed from differing clonal growth strategies and not only from differences in the vigor of clones.

Although clones growing in full light without mowing expanded laterally more than shaded ones in absolute values (Tables 1, 2), proportionally to their accumulated biomass (and thus for an equivalent vigor), the latter went further and explored more horizontal space than the former (Fig. 3a). It is therefore possible that, in shaded environments such as a forest understory, *R. japonica* displays an extensive foraging strategy to increase the chances of placing ramets in sunflecks and canopy gaps or to escape this less favorable habitat (cf. Lovett Doust 1981; Slade and Hutchings 1987a), as has been frequently reported for other species (de Kroon and Hutchings 1995; Slade and Hutchings 1987b; Xie et al. 2014). On the other hand, in a homogeneously luminous and undisturbed environment, *R. japonica* seems to adopt a space-consolidation strategy (sensu de Kroon and Schieving 1990). In this exploitative strategy, phalanx clones multiply their ramets through increased branching frequency and short spacer lengths in order to monopolize resources and limit interspecific contacts (de Kroon and Schieving 1990; Gough et al. 2001; Herben and Hara 1997; Lovett Doust 1981).

In theory, phalanx individuals should have a slower lateral expansion rate than guerrilla individuals (Lovett Doust 1981; Schmid 1986). Yet, Figure 2c and Tables 1, 2 show that *R. japonica* grew faster and explored more soil volume in 14 months when cultivated in full light than in a shady place, as long as it is undisturbed. This is consistent with observations made on cultivated *R. japonica*'s stands (i.e. composed of several competing clonal fragments) that expanded faster and further in two seasons when grown alone than when planted in mixture with a high density of *Salix viminalis* cuttings (Dommanget et al. 2019). In both cases, this difference is certainly explained by the higher vigor of clones growing in full light compared to shaded ones. Still, theory does not tell if the differences in lateral growth rates between phalanx and guerrilla individuals should be constant over time or not. It could be that, in order to operate an efficient spatial pre-emption against potential competitors, phalanx clones of *R. japonica* have a quick initial expansion rate for a while followed by a (gradual or steep) deceleration as clones get stronger and more dominant and as their chances of being excluded decline. In the long term however, guerrilla clones could perhaps expand further (to escape) or display a higher clonal mobility than phalanx ones (cf. Zobel et al. 2010). More long-term empirical studies are needed to verify these assumptions and more generally, to assess the differences in lateral growth rates between clonal fragments of *R. japonica* growing in differing environments as no data actually exist on the matter.

Against our expectations, average ramet densities and branching frequencies of entirely mowed clones in full light (M treatment) were not significantly higher than those of illuminated but un-mowed ones (L treatment), despite interesting trends. Moreover, entirely mowed individuals had an overall very low spatial expansion. This discrepancy between our hypothesis and observations is likely due to the intensity of mowing events. As these clones had to cope three times with the total destruction of their aerial organs during their first growing season (and one more time at the beginning of the next one), their biomass production and spatial exploration must have been strongly constrained (Fig. 2; Table 2), hence limiting our ability to properly observe their clonal growth patterns in less disturbed environments. The intellectually appealing hypothesis stipulating that mowing breaks the apical dominance of *R. japonica*'s ramets and thus favors rhizome branching and the lateral expansion of clonal fragments (cf. Bashtanova et al. 2009; Beerling 1990) consequently requires further study.

Stands of *R. japonica* frequently grow in habitats that do not experience full sun, or are mowed, such as roadsides, semi-natural riverbanks or forest edges (Beerling et al. 1994; Martin et al. 2019; Tiébré et al. 2008). In those, we may expect clones to adopt tactics to cope with or to avoid the effect of less favorable areas. Unfortunately, at the half-pot scale, our clones grown in heterogeneous conditions did not demonstrate many significant differences between favorable (full light and undisturbed) and unfavorable (shaded or mowed) habitat patches for most studied traits. Yet, despite this overall lack of statistical significance, clone parts growing in the favorable habitat patches of our heterogeneous treatments appeared to have produced more rhizome branches (per unit of rhizome length), more ramets, and to have accumulated more rhizomatous biomass than parts growing in unfavorable patches (Figs 2c, 4b, 5b). These observations suggest that resources are preferentially invested locally and that the un-disturbed and un-shaded parts of clonal fragments do not support much the development of parts growing in less favorable conditions, possibly demonstrating early stages of habitat selection. Evidence from previous studies already suggested that in homogeneous conditions, the level of clonal integration between ramets of *R. japonica* varied with the level of resources (Price et al. 2002; Suzuki 1994). Our results show that environmental heterogeneity may also affect patterns of resource sharing in this taxon. Additionally, shaded patches of the LS treatment harbored parts of clones that seemed to exhibit higher specific spacer lengths (Fig. 3b), which could be evidence of a trade-off between phalanx and guerrilla growth forms (e.g. Ye et al. 2006) and thus, of a localized escape strategy.

The absence of clearer morphological and architectural responses in the heterogeneous treatments may be simply linked to the methodological constraints related to the cultivation of giant herbaceous species such as *R. japonica*: i.e. small sample size and short duration of experimentation. A longer experiment, with a harvest at the end of the second growing season could perhaps have given different results, for instance for the significance of observed differences or bud bank's distribution (cf. Gao et al. 2012; Ott and Hartnett 2015; Watson et al. 1997). Further research on this topic would be useful to draw more definitive conclusions. Of course, we cannot rule out the possibility that *R. japonica* is purely unable to select a preferential habitat and that it does not attempt to escape through directional growth (e.g. Evans and Cain 1995; Sampaio et al. 2004) or selective placement of ramets (e.g. de Kroon and Hutchings 1995; Wijesinghe and Hutchings 1997).

In addition to increased sample size, it would be interesting if future experiments could increase the number of sampled populations. *Reynoutria japonica* is indeed known to be represented by the same single clone throughout most of its introduced range (Bailey et al. 2009). As such, sampling a single location was satisfactory to account for its genotypic variability, but not for potential epigenetic variations. Sampling populations along various environmental gradients could therefore potentially reveal different growth patterns (e.g. Richards et al. 2012; Zhang et al. 2017) that could help refining and expanding observations made in the present paper.

Establishment potential and management implications

Although this experiment did not aim at investigating the establishment potential of *R. japonica*, it is enlightening to observe that the thirty regenerating plants survived their first winter and were still growing after 14 months. It is even more interesting when we consider that some had to grow under heavy shade or in a frequently mowed environment. It confirms that three mowing events per year is not sufficient to kill regenerating clones of *R. japonica* (Seiger and Merchant 1997), though that may depend on the size of the propagule from which a plant is regenerating.

The vegetative propagules that we planted had a fresh weight of approximately 16 g, which represents rhizomes with a length of 12–13 cm for a diameter of 1.2 cm. Such dimensions are certainly not infrequent in the wild where *R. japonica* can annually produce underground biomass exceeding 10 $t \cdot ha^{-1}$ (Callaghan et al. 1981; Palmer 1994). Even our young clones produced enough biomass to recreate dozens of such propagules (Fig. 2c). At least two recommendations for the management of *R. japonica* can be made from these observations. Firstly, monitoring campaigns should not overlook shaded habitats as clones born from vegetative propagules may have established there. Secondly, early control campaigns should either favor the manual extraction of the whole regenerating ramets (e.g. Barthod and Boyer 2019), or remove or spray above-ground plant parts at a high frequency, to have a chance at eradicating newly established *R. japonica*.

Interestingly, clones that experienced only partial mowing (LM treatment) did not produce a significantly lower total biomass than un-mowed individuals (L treatment). Yet, the contrast with the biomass production of entirely mowed clones (M treatment) is striking (Fig. 2). It thus appears that clone parts growing in un-mowed halves of the LM treatment managed to compensate for the loss of their mowed counterparts. This is important from a management perspective as it emphasizes the need to mow/cut *R. japonica* individuals over their whole cover to truly impact their growth dynamics.

This observation gives a quantitative explanation to empirical and modelling studies indicating that low mowing frequency and/or partial mowing have close to no effects on the cover or expansion dynamics of *R. japonica* (Gerber et al. 2010; Lavallée et al. 2019; Martin et al. 2019).

Restoration of competitive native species has been shown to be a promising management solution to limit the performances and spread of *R. japonica* (Dommanget et al. 2015; Dommanget et al. 2019; Skinner et al. 2012). Control by restoration is notably interesting as it is thought to have low environmental impacts and pecuniary cost in the long-term (Dommanget et al. 2019). This kind of restoration using mostly plantings of local species to shade *R. japonica* is frequently associated with mowing during the first years of installation. In this context, it would therefore be very interesting to test the combined effect of shade and mowing on the long-term spatial dynamics of both regenerating and established clones. Besides, it would also be relevant to study the effect of other aspects of competition (not only for light) on the spatial exploration of knotweed clones. Long term studies will additionally be required to observe if *R. japonica* would be able to escape the cover of the planted native species through directional growth or spatial exploration.

Conclusion

To the best of our knowledge, this is the first time that quantitative observations of clonal growth and expansion dynamics in *R. japonica* are provided for differing environmental conditions. We believe that our results help improve our understanding of the invasion dynamics of this species at the local scale, highlighting aspects of its resilience and effects on invaded communities that will be useful for the management and modelling of this taxon. However, more research is needed to complete our results and to extend them to other knotweed taxa as well as to other epigenotypes of *R. japonica*.

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