RESEARCH ARTICLE



The distribution and impact of an invasive plant species (Senecio inaequidens) on a dune building engineer (Calamagrostis arenaria)

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Abstract

Disturbance is thought to enhance the probability of invasive species establishment, a prerequisite for naturalisation. Coastal dunes are characterised by disturbance in the form of sand dynamics. We studied the effect of this disturbance on the establishment and spread of an invasive plant species (*Senecio inaequidens*) in European coastal dunes. Local sand dynamics dictate the spatial configuration of marram grass (*Calamagrostis arenaria*). Therefore, marram grass configuration was used as a reliable proxy for disturbance. Since marram grass plays a crucial role in natural dune formation, we evaluated the possible effects *S. inaequidens* could have on this process, if it is able to naturalise in European coastal dunes.

We expected the highest probability of *S. inaequidens* establishment at intermediate marram grass cover because too low cover would increase sand burial, whereas high cover would increase competition. However, our results indicate that *S. inaequidens* is quite capable of handling higher levels of sand burial. Thus, the probability of *S. inaequidens* establishment was high under low marram cover but slightly lowered when marram cover was high, hinting at the importance of competition.

We expected a negative impact of *Senecio*-altered soils on marram grass growth mediated by soil biota. However, marram grass grew better in sand gathered underneath *Senecio* plants due to abiotic soil modifications. This enhanced growth may be caused by *Senecio* leaf litter elevating nutrient concentrations in an otherwise nutrient-poor substrate. If such increased plant growth is a general phenomenon, further expansion of *S. inaequidens* could accelerate natural succession in European coastal dunes.

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Keywords

Ammophila arenaria, disturbance, Marram grass, Narrow-leaved ragwort, naturalization, plant-soil feedback, pyrrolizidine alkaloids, South African ragwort

Introduction

Due to human activity the number of invasive species worldwide is ever-increasing. In Europe alone, the number was estimated to be well over 12 000 in 2019 (Roy et al. 2019). The causes of introduction of non-native species range from intentional introduction, e.g., for pest control, horticulture, coastal defence and restoration purposes (Cox 1992; Beckstead and Parker 2003; Richardson and Rejmánek 2011; Camacho-Cervantes et al. 2017; Buerdsell et al. 2021) to unintentional introduction such as escape from planting sites and introduction via tourism (Davenport and Davenport 2006).

After reaching a new habitat, the non-native species needs to establish and naturalize in order to become invasive (Richardson et al. 2000). It is generally thought that disturbance benefits establishment of non-native species and that it could even facilitate the spread of invasive species. Several mechanisms, such as reduced competition, increased resource input and increased habitat availability, are proposed to be behind this phenomenon (Mack et al. 2000; Pyšek and Richardson 2006; Jauni et al. 2015; Lear et al. 2020).

Several hypotheses have been proposed to explain the long-term success of invasive species (Mack et al. 2000; Chabrerie et al. 2019). Among these, the enemy release hypothesis (**ERH**) and the Evolution of Increased Competitive Ability hypothesis (**EICA**) are particularly important in the context of harsh, temporally variable environments in which biotic interactions can be hampered. The ERH states that the success of invasive species can be attributed to the release from natural enemies such as (specialized) herbivores or pathogens (Keane and Crawley 2002). The EICA adds to this that, due to the release from natural enemies, invasive plant species can reallocate resources otherwise used for protection towards growth and other performance traits (Blossey and Notzold 1995).

Invasion can also be promoted via both intra- and interspecific facilitation (Jordan et al. 2008; Proença et al. 2019; Uyà et al. 2020), especially in harsh environments, or by decreasing fitness of native species (Jordan et al. 2008; Vilà et al. 2011). One underlying mechanism in plant communities is modification of the soil (Aldorfová et al. 2020). The effect of such modifications can be very useful because invasive species can provoke generic effects against the whole native community, which enables them to invade if they suffer less from their created disaster. Contrastingly, it is hard for the native community to specifically target a newly arrived, invasive species (David et al. 2017). The term 'plant-soil feedback' (PSF) refers to the process of plants altering the soil with effects on the performance of other plants subsequently growing in this soil (Bever et al. 1997; van de Voorde et al. 2011; Buerdsell et al. 2021). The soil characteristics altered can be biological, chemical or structural (Ehrenfeld et al. 2005; Kulmatiski et al. 2008). Biological modification of the soil occurs via changes in the soil community, including soil microbes and soil fauna. Depending on the affected species, these

effects can be negative, e.g., when root-feeding nematodes or pathogens accumulate (Van der Stoel et al. 2002; Bever et al. 2015) or mutualistic interactions are disrupted by the non-native plant (Callaway et al. 2008; Brouwer et al. 2015), or positive, e.g., by accumulation of mycorrhizal fungi or nitrogen-fixing bacteria in low-nutrient soils (Rodríguez-Echeverría et al. 2009; in 't Zandt et al. 2019). Root exudates, litter decomposition and root-supported microbial activity can alter different components of the soil chemistry such as soil acidity and nutrient availability (e.g., Lazzaro et al. 2014). Structural modification occurs via changes in soil temperature, water content or the overall soil structure and soil aggregates (Ehrenfeld et al. 2005). Again, these effects can be either negative or positive, depending on the species (Bezemer et al. 2006).

Plant-soil interactions can affect the process of species invasion at different scales. Plant-soil interactions are local and thus mainly affect the plant itself or other plants in the near vicinity, both conspecifics and heterospecifics. Invasive tree species can, however, have more wide-ranging effects using their fallen leaves as agents of soil change (e.g. Gómez-Aparicio and Canham 2008). At larger spatial scales, different local plantsoil interactions give rise to a heterogeneous, spatially structured landscape (Bever et al. 1997; Mack and Bever 2014) which influences biodiversity, population dynamics and ecosystem functioning (Levine et al. 2003; Vilà et al. 2011; Mack and Bever 2014). This heterogeneity can, in turn, influence the processes facilitating species invasions, such as enemy release or fitness decrease in native competitors.

European marram grass (Calamagrostis arenaria (L.) Roth, formerly Ammophila arenaria) is one of the most extensively studied systems regarding PSF, with studies investigating abiotic and biotic PSFs going back to the 60s (Marshall 1965) and 80s (Van der Putten et al. 1988) respectively. This study focuses on marram dunes (Natura 2000 habitat 2120, CORINE biotope 16.21), a coastal habitat type dominated by marram grass, which occurs relatively early in the dune succession, characterised by high levels of stress (Kulmatiski et al. 2008) due to e.g. sea spray and aeolian sand burial (Brown et al. 2018). Marram grass is perfectly adapted to grow in these conditions. Several studies have shown that marram grass even needs sand burial to grow optimally (i.e. Nolet et al. 2018; Ievinsh and Andersone-Ozola 2021) because the biological soil community accumulating around the roots of marram grass has a negative impact on its performance and growth (Van der Putten et al. 1988; Van der Stoel et al. 2002). Deposits of sand blown in from the beach are relatively free of root pathogens and parasites and thus enable marram grass to develop new roots in this temporarily enemy-free soil. Additionally, marram grass also interacts with the aeolian sand dynamics by locally lowering the wind speed and thus promoting sand capture (Zarnetske et al. 2012; Reijers et al. 2021), resulting in a positive feedback between marram growth and sand capture. This interplay between marram grass and sand dynamics leads to a range of possible spatial configurations of marram grass, which depend on the local sand dynamics. Reijers et al. (2021) found that marram grass grows highly clustered together under sediment-poor conditions. When there is enough sediment supply, it grows more randomly, albeit still clustered (Reijers et al. 2021). This allows marram grass cover to be used as a proxy for the intensity of sand dynamics. Marram grass can reach a high density when enough fresh sand is provided by strong sand dynamics.

Under moderate sand dynamics, an optimal spatial configuration is expected to occur with a heterogeneous mosaic of bare sand and marram grass. Since this species grows via lateral vegetative growth, natural configurations show variable degrees of clustering, but rarely occur in truly random, let alone regular configurations.

The bare sand patches between marram grass tussocks may provide an opportunity for invasive species to establish. On the other hand, too dynamic conditions will probably hinder settlement due to too high levels of sand burial (Maun 1998; Kent et al. 2005). These conflicting pressures could determine where invasive species are able to establish in marram-dominated dunes. If these sand patches become overgrown, sand dynamics can further decrease (Gao et al. 2020) and in turn this can negatively affect the vitality of marram grass. This could lead to the invasive species becoming competitively stronger and outcompeting marram grass (i.e., a form of positive density dependence). Such changes in competition could have extensive consequences for coastal dunes and their ecosystem services (Klironomos 2002), especially coastal defence, as they could trigger feedbacks that change system dynamics (Bonte et al. 2021).

One species invading coastal dunes around the North Sea is narrow-leaved ragwort (*Senecio inaequidens* D.C., Asteraceae, also known as South African ragwort). It is originally a South African species, but with a long history of invasion in Europe (Ernst 1998), where it arrived via wool transport (Lachmuth et al. 2010). Although much is known about the invasion of *S. inaequidens* in other habitats in Europe (Ernst 1998), far less is known about its colonisation of sandy dune areas. It was first found in dune areas in 1935, more specifically in the dunes of Calais, France (López-García and Maillet 2005).

Senecio species contain pyrrolizidine alkaloids (PA) as a defence mechanism against both above- and belowground herbivory (Joshi and Vrieling 2005; Caño et al. 2009; Thoden et al. 2009; Joosten and Van Veen 2011). Several studies have shown these allelopathic defences can influence entire soil communities (Kowalchuk et al. 2006; Thébault et al. 2010; Harkes et al. 2017) and therefore *Senecio* species are able to affect their own spread (Engelkes et al. 2008). However, the exact mechanisms are, to our knowledge, still unknown. Passive release from roots (and leaf litter) is the most probable pathway, although it is speculated that direct secretion from the roots is possible as well (Kowalchuk et al. 2006; Joosten and van Veen 2012; Selmar et al. 2019). Not many studies have tried to investigate the direct effect of PAs on plant growth (but see Ahmed and Wardle 1994). Recently, even uptake of PAs by other plant species was demonstrated (Nowak et al. 2016; Selmar et al. 2019), although the general consequences of this horizontal transfer for the receiving plants are unknown at the moment.

We suspect that PAs in sandy soil will have little effect on marram grass growth directly. The sign of the total effect of *S. inaequidens* will depend on the response of the soil community. It will be negative if marram pathogens can accumulate or if PAs prevent symbionts from associating with marram roots. However, it can be positive if PAs prevent accumulation of marram pathogens and thus create an enemy-free space for marram roots, as aeolian sand does.

Here, we investigate the relation between marram grass spatial configuration and the probability of establishment of *Senecio inaequidens* in marram dunes, together with the potential effects of this invasion on marram dunes. We hypothesize that (1) due to

the potentially positive effect of disturbance on invasive species (Scherber et al. 2003; Jauni et al. 2015), *S. inaequidens* will likely get established in more disturbed areas, i.e. areas with stronger sand dynamics. However, since too high sand burial is probably detrimental for the growth of *S. inaequidens*, we expect to find an optimum at intermediate sand burial which is also associated with intermediate vegetation cover. We further postulate that (2) the biotic compartments of *Senecio*-altered soils will negatively affect marram grass growth, except if PAs prevent marram pathogens from accumulating.

Material and methods

Study area

This study was carried out in coastal dune areas along the Channel and the North Sea, covering the North of France, Belgium, the United Kingdom and the Netherlands (Fig. 1). Within this area, we focussed on sandy coasts with marram-dominated, yellow dunes. This area included the location of *S. inaequidens* settlement and the northern-most location within its distribution in coastal dunes, thus enabling us to study the front of the ongoing invasion.



Figure 1. The samples included in the analysis. Colours indicate the different countries. *Senecio inaequidens* was not found in the UK. Map made with QGIS v3.6 (QGIS Development Team 2021).

For a recent biodiversity study, 46 dune transects spread along the study area were selected. The transects had a mean length of 1212 m (shortest: 230 m, longest: 3348 m) and were located within the first 100 m from the front of the foredunes. Within each transect a number of sampling locations was chosen based on the length of the dune transect with an average of 14 samples (min 5; max 37). Each sampling locations were separated by a central marram grass tussock. Individual sampling locations were separated by at least 20 m and chosen with the aim to maximise the variety of surrounding marram grass configurations. For the total number of samples and transects per country, see Table 1.

Country	Samples	Transects	Mean length of transects
BE	206	18	822
FR	184	9	2232
NL	188	13	800
UK	60	6	720

Table 1. The number of samples taken in each country within the study region.

Data collection

The occurrence of narrow-leaved ragwort (*Senecio inaequidens*) was mapped at each sampling location. The number of *S. inaequidens* plants was counted within a radius of 5 m around the central marram grass tussock for those sampled in France, the UK and the Netherlands. Due to a change in the protocol of the biodiversity study, in Belgium the occurrence was scored into four categories: "not present", "sparse", "moderate" and "abundant". Data on the occurrence of *S. inaequidens* were collected during three consecutive summers: in July 2017 data were collected along the Belgian coast; in July, August and September 2018 along the French coast; in August and September 2018 along the UK (Norfolk and Devon).

From available vegetation maps of the foredunes (Bonte et al. 2021), the proportional cover by marram grass in the vicinity of the central marram grass tussock (P), together with a measure of spatial autocorrelation of marram grass occurrence (normalised join count statistics, JC; Cliff and Ord 1981), were calculated. These two parameters were used to express the spatial configuration of the surrounding marram grass. The proportion of marram grass cover is straightforward and ranges from 0 (no marram grass present) to 1 (the whole area is covered with marram). The measure of spatial autocorrelation is negative when the marram grass is regularly distributed in the landscape. If the marram grass is randomly distributed, the parameter is close to 0 and it is positive when the marram grass occurs clustered together. As pointed out by Bonte et al. (2021), marram grass distribution is almost always clustered (i.e., high JC values) and rarely random. These two parameters (P and JC) were calculated within four circles with different radii (5 m, 10 m, 20 m, 50 m) around the central marram grass tussock to represent different spatial scales. See supplementary material of Bonte et al. (2021) for a more in-depth explanation of construction of the vegetation maps and calculations of the spatial parameters.

Lab experiment

To study the effect of *S. inaequidens* on marram grass growth, we performed a growth experiment with a split-plot design: sand affected by *S. inaequidens* was gathered at the Belgian coast together with bare sand for the control group. Half of the volume of sand gathered was sterilised (by autoclaving at 121 °C/1 bar for 30 minutes) in both groups to determine whether any observed effect could be biotic or abiotic.

Sand was gathered from three different sites situated on the western, central and eastern Belgian coast: in the foredunes in Oostduinkerke (Ter Yde) for the west coast, for the mid coast in Oostende (Fort Napoleon) and for the east coast between Wenduine and Zeebrugge (two locations were used due to low occurrence of *S. inaequidens*). Ten plots were sampled at each site (for a total of 30 plots). Each plot yielded two samples: 2L rhizospheric sand from underneath *S. inaequidens* plants and 2L of bare sand taken 5–10 m away. This way, changes in soil between two paired samples, other than due to the influence of *S. inaequidens*, were minimised. The sand was stored in the fridge (max 3 days) to assure the survival of the soil biota until the sand was used. The 2L samples were divided into two 1L sub-samples from which one was sterilised and the other was not. Thus, we had four treatments: *Senecio*-influenced vs. bare sand at the plot level combined with sterile vs. non-sterile soil at the subplot level (Fig. 2). In other words, influence of *Senecio* was the whole-plot factor and soil sterilisation the subplot factor, with whole plots organized in pairs, which act as statistical blocks.

Marram grass seedlings were used for the experiment because seedlings are more susceptible to environmental influences than fully grown plants (Huiskes 1979). The seedlings were grown from seeds gathered at the Belgian coast (Oostduinkerke, ter Yde) from the same population in order to minimize genetic effects. The seeds were collected during the summer of 2019 and stored at room temperature in the lab. All seeds were surface-sterilised as in de la Peña et al. (2010) before they were left to germinate under standardized conditions (on commercially available sand saturated with demineralized water; photoperiod: 16/8 h light/dark; temperature: 22 ± 1 °C) for 2 weeks prior to dune sand collection. As a baseline, the whole seedlings were weighed and the length of roots and leaves was measured before planting.

All 120 pots (3 sites \times 4 treatment combinations \times 10 plots) were filled with 1L of sand in which three seedlings were planted. The pots were placed in a growing chamber under the same conditions as mentioned before for the germination of the seeds. All pots were watered twice a week, on the same day, with demineralised water until near-saturation. Each pot was labelled with a unique ID in order to prevent observer bias.

After 2 weeks of growing, the largest seedling was selected to grow for another 10 weeks. The other two seedlings were removed. This was done to ensure that all remaining seedlings had rooted properly in order to minimise die-off and resulted in only



Figure 2. Split-plot design of the growth experiment for the site at Oostduinkerke (Western Belgian coast). 2L samples of sand, (1) sand from unvegetated locations or (2) sand from underneath *Senecio*, were split into two 1L subsamples, one of which was sterilized. This enabled us to investigate whether the effect of *Senecio* was achieved via the biotic or abiotic portion of the soil. Map made with QGIS v3.6 (QGIS Development Team 2021). Aerial photograph (summer 2018) source: Agency for Information Flanders (geopunt.be).

three plants dying during the whole experiment (one from each treatment, except for the sterilized bare sand treatment). At the end of the growing period the whole plants were collected, all leaves were counted and the length of the longest leaf and root was measured. Further, all leaves and roots were weighed separately, both before and after drying in an oven at 70 °C for 48 h.

Statistical analyses

Occurrence of Senecio

Due to two different methods of assessment of the occurrence of *Senecio inaequidens* (i.e. ordinal categories for the Belgian samples and count data for all other samples), all *S. inaequidens* data were converted to presence/absence. To exclude false zeros (i.e., samples along dune sites where *S. inaequidens* is not yet established) from the analysis, only dune transects where *S. inaequidens* occurred in at least one sample were included. This resulted in a final dataset comprising 26 out of the 46 original sites, which included 408 of the original 638 samples. The sites were located in three countries since *S. inaequidens* was not observed in the United Kingdom.

The marram grass spatial data were used as independent variables. As explained above, the spatial data consisted of two continuous variables: the proportion of marram grass (P) and its normalized join count statistic (JC) for each spatial scale (5 m, 10 m, 20 m and 50 m) per sample. The JC values were rescaled to the maximum value to alleviate convergence issues of linear models. This resulted in both parameters ranging between 0 and 1. Generalised linear mixed models were used with a logit link function and binomial distribution to analyse the occurrence data. A combination of first and second order terms of P and JC, together with interactions between them, were fitted to allow the relationship between the occurrence of *S. inaequidens* and the spatial parameters to be unimodal. The maximal (full) generalised linear mixed models were of the form:

To determine which combination of P and JC best explained the occurrence data, different combinations of the spatial predictors were fitted (including interactions terms, see Suppl. material 1: Table S1 for all models) at all four scales (i.e. using P and JC computed at 5 m or 10 m or 20 m, etc.), after which model selection based on the corrected Akaike Information criterion (AICc) was used to select the model and scale that optimised goodness-of-fit. Dependency is present within the data for samples along the same transect. Therefore, 'transect' nested within 'country' was included in the models as a random variable. 'Country' itself was excluded because it contained almost no variation (Chen and Dunson 2003). This way we also accounted for differences in weather, dune management and time (different countries were sampled in different years).

Growth experiment

We analysed the effect of the provenance of the sand (from beneath *S. inaequidens* vs. bare sand), of its sterilisation and of their interaction using linear mixed models. F-tests with Satterthwaite's approximation of denominator degrees of freedom were used to determine the significance level of the fixed effects. All measured traits (number of leaves, length of longest leaf and root, weight of fresh and dry roots and leaves) where highly correlated (see Suppl. material 1: Fig. S1, Table S2), so we used the first principal component (PC1) from a principal component analysis run on the trait data as response variable for the analysis. Sample site and plot were integrated in the mixed model as random effects to account for data dependency within block and whole-plots. Sample was initially also included to correct for dependency of the subsamples within each sample, but this random effect was removed because of a negligible variance component.

All data analyses were performed using R Statistical Software (R Core Team 2021). The calculation and normalisation of JC values was done with the 'spdep' package (Bivand and Wong 2018). The packages 'lme4' (Bates et al. 2015) and 'lmerTest' (Kuznetsova et al. 2017) were used for the Generalized linear mixed models. Package 'MuMIn' (Barton 2020) was used for automated model construction and comparison.

Results

Occurrence

Senecio inaequidens was observed at 176 of the 408 sites included in the analysis. The most northern and southern transect where *S. inaequidens* was observed are respectively at Wassenaar (52.1565°N, 4.3404°E; the Netherlands) and Wimereux (50.7931°N, 1.6074°E; France). *S. inaequidens* was most frequently present in Belgian samples, followed by France and the Netherlands (Fig. 3). In the United Kingdom, no *S. inaequidens* was observed.

The four models selected were all at the 5 m scale (using an AICc delta value of 2; see Table 2 for the selected models; see Suppl. material 1: Table S1 for all models), implying that *S. inaequidens* reacts to marram grass spatial configuration at small distances. The predicted occurrence is depicted in Fig. 4a. When the vegetation is highly clustered together (high JC values), the occurrence of *S. inaequidens* is negatively correlated with marram grass cover. Further, we see a clear minimum probability of occurrence of



Figure 3. The average occurrence of *S. inaequidens*, calculated as the proportion of samples within each transect where *S. inaequidens* was found. BE = Belgium; FR = France, NL = the Netherlands.

Table 2. The coefficients, number of model parameters (df), AICc values, relative AICc (Δ AICc; i.e., difference between each model's AICc and the minimum AICc) and Akaike weights for all selected models.

Spat.	Intrcpt	JC	JC ²	Р	P ²	JC*P	JC*P ² JC ² *P	df	logLik	AICc	Δ	weight
scale											AICc	
5	-1.4	6.13		-8.84	15.75		-13.71	6	-182.35	376.96	0	0.13
5	-2.53	15.39	-11.49	-24.36	28.00	23.84	-32.06	8	-180.9	378.24	1.285	0.068
5	-3.14	12.46	-5.35	-8.92	14.82		-12.19	7	-181.99	378.32	1.367	0.065
5	-0.12	3.94		-16	22.28	11.32	-23.80	7	-182	378.34	1.379	0.065
Avg.	-1.73	8.88	-3.45	-13.50	19.40	7.20	-19.21					

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Figure 4. a The overall relation between the probability of occurrence of *S. inaequidens* and the spatial configuration of marram grass. The colours indicate the probability of occurrence as %. **b** Density distribution plots of the observed cover (P) and spatial autocorrelation (JC) of marram grass within a 5 m radius of the central marram grass tussock. This plot only contains the data of the transects where *S. inaequidens* was found. Colours indicate whether *S. inaequidens* was present (yellow) or absent (purple).

S. inaequidens at more random distributions of marram grass (low JC values) with intermediate vegetation cover. Since no random configurations were found at intermediate P, the minimum is probably due to a small number of samples with a low JC (see Fig. 4b), thus caution is advised when interpreting this result. Although we actively tried to sample in a wide range of different spatial configurations of marram grass, there is still a low number of data points with low marram grass cover and less clustered configurations due to the nature of the system. To ascertain that the outcome of the analysis was not greatly influenced by those few samples, the analysis was redone after excluding those samples. The results did not differ greatly (see Suppl. material 1: Fig. S2).

Growth experiment

The first PC of the PCA of all measured plant traits explained 73.9% of the variation, while the second PC explained 14.7%. Scores along PC1 were significantly correlated with all plant traits (see Suppl. material 1: Fig. S1, Table S2), therefore, we used PC1 as a reliable indicator of overall plant growth. Marram grass growth was affected by both the abiotic and biotic components of the soil (Fig. 5). However, no significant interaction was found ($F_{1,82.7} = 1.10$, p = 0.298). Soil sterilisation had a positive effect on marram growth ($F_{1,83.4} = 106$, p < 0.001), which means that the soil biota had a negative effect on marram biomass. The plants grown on sand from underneath *S. inaequidens* grew better than plants grown on bare sand ($F_{1.82.7} = 59.2$, p < 0.001).

Discussion

Field data

No evidence was found for the hypothesized optimum probability of establishment of *S. inaequidens* at intermediate marram grass densities. In fact, our results indicated that



Figure 5. Box- and violin plots represent distribution of PC1 values for marram grass growth. Horizontal lines above the boxplots indicate comparisons between treatments, *** indicate significant difference of p < 0.001. Colours indicate whether biota were present (yellow) or absent (purple). Number of samples per treatment is 29, except for sterilized sand from unvegetated locations, where it is 30.

S. inaequidens has no problem growing in sandy conditions, as we observed a negative correlation between vegetation cover and probability of establishment. This indicates that *S. inaequidens* is more susceptible to competition than to sand burial. Indeed, some studies found that this species is a good coloniser rather than a good competitor (Scherber et al. 2003; Caño et al. 2007; Thébault et al. 2011). Furthermore, the available area to root in – in this case open sand – also decreases as vegetation cover increases. This is especially important since *S. inaequidens* is an annual plant which relies on high propagule pressure to spread (Thébault et al. 2011).

Due to the nature of the system, higher proportions of marram grass occur mainly towards later stages of succession. In these later stages, marram starts to decay and the spatial configuration starts to return to a more random distribution (i.e. lower JC values and slightly lower P values) because marram grass is slowly being replaced by other plant species. This leads to a rise of the probability of *Senecio* establishing which may indicate that it is becoming a stronger competitor.

Overall, the probability of establishment of *S. inaequidens* displays high values across the whole range of sampled natural marram grass configurations. Since we aimed to maximise the variety of natural marram grass configurations surrounding the sample, configurations that were not sampled probably do not, or not often, occur in nature. In fact, such configurations arise probably mainly when marram grass is planted (i.e., for coastal protection) and afterwards when the planted dune is 'maturing'. This makes it hard to extrapolate our findings to these specific situations.

Growth experiment

We hypothesised that the effect of *S. inaequidens* on marram grass growth would be negative, mainly because of interactions with the soil community. However, we concluded that the overall effect is positive. This effect is purely abiotic, since there is no significant interaction between sand sterilisation treatment and the provenance of the sand (underneath/away from *Senecio*). Similarly, intraspecific plant-soil feedbacks from *Senecio jacobaea* are also known to be (partly) abiotic (Wang et al. 2019), although the effect was negative in the cited study. Dassonville et al. (2008) showed that invasive species (*S. inaequidens* being one of the species included in their review) can have a positive effect on nutrient concentrations in plots with initially low concentrations. Since sand indeed has low nutrient concentrations (Reijers et al. 2020), this explanation is applicable here. Currently, we cannot verify this explanation since nutrient concentrations were not measured in the experiment.

Because marram grass growth was promoted in sand influenced by *S. inaequidens*, we can conclude that pyrrolizidine alkaloid concentrations had no, or a negligible, negative effect on marram grass. This is not surprising, since the most probable mechanism of PA enrichment of the soil is via passive release from roots and leaf litter (Joosten and van Veen 2012; Nowak et al. 2016; Selmar et al. 2019) and because some plants are even known to take up PAs without experiencing adverse effects (Nowak et al. 2016; Selmar et al. 2019). In contrast, Ahmed and Wardle (1994) found a negative effect of PA on plant growth. In our study, such a negative effect of PAs may have been counteracted by the simultaneous nutrient enrichment caused by *S. inaequidens*. This is in line with the observations by Reijers et al. (2020) that marram grass is more capable of coping with stressful conditions when nutrient availability is higher.

We observed a significant negative effect of soil biota on marram growth, with sterilisation of the soil having a positive effect on the biomass of marram, independent of the sand origin. This indicates that soil biota in the Senecio rhizosphere have approximately the same (negative) effect as the community within unvegetated sand. Thoden et al. (2009) found that PAs suppress the development of juvenile Meloidogyne hapla nematodes. Species from this genus also colonise marram grass. However, it was suggested before that nematodes from this genus do not develop to adults on marram grass anyway (Van der Stoel et al. 2002). On the other hand, Pratylenchus nematodes are able to colonise both Senecio (Zasada et al. 2017) and marram grass roots (Van der Stoel et al. 2002), which would enable infection of marram roots by Pratylenchus spp. present on Senecio roots. However, arbuscular mycorrhizal (AM) fungi have low levels of endemism and host specificity (Davison et al. 2015; Aldorfová et al. 2020) and are known to colonise roots of Senecio spp. (van de Voorde et al. 2010; Alguacil et al. 2012; Reidinger et al. 2012). Since both European and American marram grass (Calamagrostis arenaria and C. breviligulata resp.) benefit from AM fungi when faced with nematode infection (Little and Maun 1996; De La Peña et al. 2006), it is possible that the negative effect of the nematodes is counteracted by the AM fungi also accrued on the Senecio roots. Furthermore, some studies have shown that Senecio species effectively reduce density or diversity, depending on the study, of whole soil communities (Kowalchuk et al. 2006;

Thébault et al. 2010; Harkes et al. 2017). However, since these studies focussed on the effect on whole soil communities, it is difficult to make predictions for marram grass specifically. We conclude that PA concentrations in the field did not reduce the overall negative effect of the soil community, either because the community as a whole was affected and thus both negative and positive elements therein were reduced or because PA concentrations are too low to affect the soil community in general.

Our results indicate that the biotic soil community surrounding *Senecio* roots has approximately the same (negative) effect as the community within sand without plants growing in it (i.e., no significant interaction effect). Since endoparasites are known to be more damaging to marram grass (Van der Putten and Van Der Stoel 1998), it could be that we excluded their effect because we did not use an inoculum from *Senecio* to infest the soil and consequently marram roots with endoparasites. On the other hand, the biota accumulating on the senecio roots could not be compatible with marram grass roots at all. Consequently, sand from unvegetated patches, which is thought to function as a temporary 'enemy-free' space for marram grass to root in (Van der Stoel et al. 2002), has the same biological effect on marram growth as *Senecio*-influenced sand.

The observed positive effect of sterilisation in the unvegetated sand is caused by soil biota, such as nematodes, who have survival stages that can disperse in the dunes (e.g., *Heterodera* cysts) and subsequently colonise the marram grass roots in the lab (e.g., De Rooij-Van Der Goes 1995; Van der Stoel et al. 2002). Indeed, studies investigating marram grass PSFs frequently used sand from the beach or even the sea floor for their control treatment (Van der Putten et al. 1988; Van der Putten and Troelstra 1990). However, we decided to take sand from the foredunes in order to maximise similarity of soil characteristics between *Senecio*-affected and unvegetated paired samples.

Since we only studied correlations, it could be that *S. inaequidens* established only on the more nutrient-rich sand in the dunes, which would in turn explain why marram grass grows better in this sand. However, this is very unlikely since dunes are extremely dynamic and hence the top layers of sand are thoroughly mixed, creating a homogenously resource-poor environment (Reijers et al. 2020). Furthermore, marram grass was growing in the dunes long before *Senecio*, thus, if there would be patches with more nutrients, those patches would likely already have been occupied by marram grass. When sand is fixated by plant roots, the mixing is halted and nutrient heterogeneity can start to occur. Since we took sand from the rhizosphere of *S. inaequidens*, higher nutrient availability caused by *S. inaequidens* is still a viable explanation for our findings.

Integration of field data and experiment

Sandy habitats, such as coastal dunes, are characterised by unstable substrate with many open patches of bare sand in between the vegetation. These patches are ideal opportunities for the establishment of new species (Axmanová et al. 2021). From our field survey, we can conclude that *S. inaequidens* is indeed capable of colonising these open patches. The results from our lab experiment further indicate that establishment of *S. inaequidens* can enhance marram growth in particular, but probably also plant growth in general, after it dies off and nutrients become homogenised. Analogous posi-

tive effects on growth of co-occurring plants have been found for other *Senecio* species (van de Voorde et al. 2011). This enhanced plant growth can lead to an overall shift towards more vegetated dunes, thus further intensifying the worldwide trend towards dune stabilisation (Gao et al. 2020). Dune stabilisation directly implies lower sediment transport to dune parts further inland, which may enable establishment of other species and hence accelerate natural succession.

For marram grass specifically, reduced sediment supply due to dune stabilisation leads to a shift towards a more clustered vegetation configuration (Reijers et al. 2021) which optimises sand capture at small spatial scales (Reijers et al. 2019). In contrast, the potential for dune formation at larger spatial scales will be reduced, affecting dune geomorphology as a whole, ultimately resulting in lower dunes (Reijers et al. 2021). If lowered sediment supply indeed also accelerates dune succession, marram grass will be replaced more rapidly by other plant species less capable of forming dunes.

In conclusion, invasion of dune ecosystems by *S. inaequidens* could lead to a shift in sand dynamics by colonising bare sand patches, in turn accelerating the natural succession of dune vegetation. This could hamper dune growth and further reduce dune height. A reduction in dune height could in turn compromise coastal protection, since higher dunes are known to better protect the hinterland (Zarnetske et al. 2012; Seabloom et al. 2013).

Data availability

The raw data are available via Zenodo at https://doi.org/10.5281/zenodo.6138540. (Van De Walle et al. 2022).

Author contributions

RVDW, MLV and DB designed the lab experiment. RVDW conducted the practical work, analysed the data and wrote the first draft of the manuscript. All authors contributed substantially to interpretation of the results and revision of the manuscript.

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Supplementary material I

Tables S1, S2, Figures S1, S2

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Data type: docx. file

- Explanation note: Occurrence data *Senecio inaequidens*: Table S1. The spatial scale, coefficients, degrees of freedom (df), AICc values and weights for all models. PCA growth experiment: Figure S1. PCA plots for growth experiment. Groups: (left) biota-treatment: red = sterilized, blue = unsterilized; or (right) *Senecio*-treatment: red = *S. inaequidens*, blue = unvegetated sand. Table S2. Correlation of all measured traits with PC1. Sensitivity analysis of result: Figure S2. The analysis of the occurrence data, rerun without samples with very low P and JC values.
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RESEARCH ARTICLE



Ecological and potential socioeconomic impacts of two globally-invasive crayfish

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Abstract

Quantifying the impacts of invasive species, relative to native analogues, is crucial for management and policy development. Two freshwater crayfish species of global concern, Cherax quadricarinatus and Procambarus clarkii, have established populations across Africa. Negative impacts on native biodiversity and socioeconomic impacts have been documented in other continents; however, there is a paucity of information on impacts from Africa and for C. quadricarinatus. To fill this literature gap, this study used laboratory experiments to determine potential ecological and socioeconomic impacts conferred by the crayfish species relative to a functionally similar native analogue, the river crab Potamonautes perlatus, on two static, but different resources. Consumption rates were derived for the three focal species consuming the macrophyte Potamogeton nodosus and dead Oreochromis mossambicus under different temperatures regimes (19 °C and 28 °C), representing summer and winter seasons in Southern Africa, with maximum feeding rate used to infer impact. Potamogeton represents ecologically-important nutrient cycling macrophytes, as well as crucial habitat for juvenile fish, whereas dead O. mossambicus was used as proxy for fish catches in artisanal gillnet fisheries often scavenged by crayfish. Consumption of both resources by all the decapods increased with temperature. However, the two invasive crayfish showed different impact trends where P. clarkii had a significantly higher consumption of macrophytes than the other two decapods regardless of temperature and the same trends seen, but for C. quadricarinatus scavenging on fish. Crayfish introductions clearly have potential for highly destructive ecological and socioeconomic impacts to invaded systems as compared to the native crabs. The disparity between resource use emphasises the necessity to use appropriate geographical and species-specific contexts to avoid erroneous conclusions from generalised risk assessments. Derived feeding rates can be used for rapid impact assessments and comparisons in other invasion cores.

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Keywords

Cherax quadricarinatus, fishery, freshwater crabs, macrophyte, Potamonautes perlatus, Procambarus clarkii, scavenging

Introduction

Invasive alien species (IAS) are widely recognised as drivers of change; thus, impetus is on predicting, quantifying and mitigating impacts across sectors whether they be positive or negative, to provide evidence for legislators (Ricciardi et al. 2013; Blackburn et al. 2014; Tickner et al. 2020; Vimercati et al. 2020). Inland waters are disproportionately at risk of invasion due to high levels of anthropogenic disturbance and lack of inclusion in major global policy and initiatives, such as the sustainable development goals, despite contributing to numerous facets, such as alleviating poverty and hunger (Lynch et al. 2020).

Ecological impacts of IAS are comparatively well described compared to other sectors, such as social or economic impacts. Yet, there remain large geographic and taxonomic gaps which must be assessed in order to compel policy-makers to prioritise IAS management (Diagne et al. 2020). African nations and rural populations globally, rely directly upon fish products for both food and nutrition security, as well as many social, cultural and economic benefits gained from the biodiverse water resources (Chan et al. 2019; Olden et al. 2020). Without suitable predictive assessments available, environmental management recommendations are often made on the basis of family level proxies or data from other geographic regions (Hawkins et al. 2015). Lack of sufficient knowledge regarding impact prediction therein puts economic, ecological and social sectors related to inland fisheries at risk of being overlooked in future policy developments, which may further exacerbate invasion impacts.

Freshwater crayfish are amongst the most notorious and destructive IAS globally (Lodge et al. 2012; Twardochleb et al. 2013; Haubrock et al. 2021). Five species of invasive crayfish have established populations in Africa (Madzivanzira et al. 2020); this is of particular concern as crayfish are phylogenetically unique in continental Africa and are, therefore, highly novel invaders (Lodge et al. 2012; Madzivanzira et al. 2020). The two most widespread and successful species: Australian redclaw crayfish *Cherax quadricarinatus* (von Martens 1868) and Louisiana red swamp crayfish *Procambarus clarkii* (Girard 1852), are spreading at a fast rate and are invasive in several ecologically- and economically-important wetlands (Madzivanzira et al. 2020, 2021c). Despite crayfish being a model ecological species and generally being shown to have broad pervasive negative impacts on both ecology and economics (Lodge et al. 2012), there are major data deficits with regards to impacts in African systems (Madzivanzira et al. 2020) and *C. quadricarinatus* impacts globally (Haubrock et al. 2021).

Crayfish impacts include the reduction of basal resources i.e. aquatic macrophytes, predation on invertebrates and reduction of amphibian and fish abundance (Twardochleb et al. 2013; Madzivanzira et al. 2021a). *Procambarus clarkii*, in particular, has been implicated as a major driver of macrophyte reduction which can cause cascading effects on fish, bird and invertebrate abundance via direct and indirect competition for resources (both habitat and energy requirements) (Grey and Jackson 2012). Macrophyte and leaf litter breakdown is a critical step in transferring energy and nutrients from basal resources to higher trophic levels (Choi and Kim 2020). Shredding behaviour by invasive crayfish is likely to accelerate macrophyte and leaf litter breakdown (Jackson et al. 2016). Large freshwater shredders are under-represented in African systems, with freshwater crabs of the Potamonautes genus (Jackson et al. 2016) presented as the closest native trophic analogue. Potamonautid crabs are predicted to be negatively impacted as a result of crayfish invasion as functionally similar species are more likely to be competitively excluded or outcompeted (de Moor 2002; Jackson et al. 2016; Dick et al. 2017). Replacement of the native crabs by invasive crayfish will considerably alter key ecosystem services, such as fishery production and water quality (Jackson et al. 2016; Madzivanzira et al. 2021a).

Human livelihoods are also affected directly by crayfish invasions. Artisanal fishermen have reported anecdotally how crayfish affect their catches through partial consumption of fish caught on static gillnets (Weyl et al. 2017; Madzivanzira et al. 2020). This has been reported for P. clarkii from Lake Naivasha, Kenya and the Nile River, Egypt and for C. quadricarinatus in the Kafue River, Lake Kariba and Barotse floodplain, Zambia, as well as in tilapia fisheries in Mozambique (Madzivanzira et al. 2020). Partially consumed fish left in the nets are not marketable as potential buyers consider the fish to be spoilt (TCM and JS, pers. obs). Owing to the significant contribution from fisheries to livelihoods as a source of protein, income or supplementary income, as well as the wider associated value chains (Aquatic Ecosystem Services and WWF 2020), the losses associated with crayfish damage pose potential for severe and escalating costs if mitigation efforts are not undertaken. The IUCN adopted protocol for assessing ecological impact [Environmental Impact Classification for Invasive Species (EICAT)] relies upon previously documented ecological impacts (Hawkins et al. 2015). Management actions are, thus, based upon their invasion history and impacts documented elsewhere (Ricciardi et al. 2013); however, this precludes the speculative assessment of novel or potential invaders (Laverty et al. 2017). Documenting field impact can often take a prohibitively long time and many resources. Various consumption rate experiments may be carried out in the laboratory to test the broad hypothesis that invasive species incur negative effects due to more efficient resource consumption relative to a native analogue (Dickey et al. 2020). In these instances, the use of a contextually and functionally relevant analogous species is integral for generating appropriate inferences.

Therefore, we quantify resource consumption by *C. quadricarinatus* and *P. clarkii* in comparison to a native analogue, *Potamonautes perlatus* feeding on two static resources: 1) Long-leaved pondweed *Potamogeton nodosus* (Poir) and 2) dead Mozambique tilapia *Oreochromis mossambicus* (Peters 1852). Both resources are economically and ecologically important to fishery productivity and value. Macrophytes constitute

the diet of most fishery species in African freshwater systems (e.g. Red breast tilapia *Coptodon rendalli*) (Weyl and Hecht 1998) and provide spawning ground and shelter for fish (Choi and Kim 2020). Consumption rates were investigated at temperatures which are representative of field conditions (19 °C and 28 °C) as temperature is a major driver of resource assimilation patterns (Uiterwaal and DeLong 2020). Based on previous studies (see Madzivanzira et al. 2021a), we hypothesise that: 1) *P. perlatus* feeding decreases with increasing temperature, 2) *C. quadricarinatus* has an equal or higher feeding rate than *P. perlatus*, regardless of temperature, 3) *P. clarkii* increases feeding with temperature, but has a lower impact than the other focal species. The study further attempts to estimate the loss in catch in the invaded regions of the Zambezi Basin.

Materials and methods

Collections of animals

Live *C. quadricarinatus* specimens were collected from sugarcane irrigation ponds in Nkomazi, Komatipoort in the Inkomati Basin, Mpumalanga Province (-25.5°S, 31.9°E). The recommended standard gear for trapping the *C. quadricarinatus* (Madzivanzira et al. 2021b) was used. The same gear was also successfully used to catch *P. perlatus* samples from dams in the Eastern Cape (-33.3°S, 26.5°E; -33.3°S, 26.5°E).

Live *P. clarkii* crayfish samples were collected from Mimosa Dam (-27.8°S, 26.6°E) in Odendalsrus, Free State Province, South Africa. In addition to the trapping method described above, rectangular traps (63.5 × 38 cm) baited with fish heads (Barkhuizen et al., accepted) were used to capture *P. clarkii*.

All animals caught were placed in 60 litre cooler boxes with fresh water from the source, with battery-powered air pumps and transported to a biosecure laboratory at the South African Institute for Aquatic Biodiversity (SAIAB) in Makhanda where they were acclimatised to the laboratory for at least a month prior to experimentation. Water temperature was maintained at 22 ± 1 °C and the laboratory was held under a 12:12 light:dark regime with white light and total darkness. Crayfish and crabs are omnivores (Geiger et al. 2005; Gherardi 2007; Souty-Grosset and Fetzner 2016) and, hence, all animals were maintained on cabbage leaves and cultured *Eisenia* sp. worms.

Prior to the experiments, all animals were acclimatised to the desired temperature at a rate of 1 °C/day and allowed to acclimatise to the two temperatures for a week before experiments were conducted. No animals were re-used per temperature treatment for both resources.

Macrophyte consumption

Potamogeton nodosus was collected from a pond in Makhanda, South Africa. *Potamoge-ton nodosus* is a heterophyllous monocotyledonous aquatic plant with both floating and submerged leaves (Ryan 1985) present in most freshwater systems in Africa (Kaplan

Species	Experiment	CL (mm)	Mass (g)
Cherax quadricarinatus	Macrophyte	60.01 ± 1.31	68.83 ± 2.82
Procambarus clarkii	Macrophyte	56.24 ± 1.14	59.63 ± 1.22
Potamonautes perlatus	Macrophyte	53.28 ± 1.16	87.72 ± 4.92
Cherax quadricarinatus	Fish	63.20 ± 1.10	67.34 ± 2.52
Procambarus clarkii	Fish	58.62 ± 1.53	59.54 ± 1.58
Potamonautes perlatus	Fish	53.27 ± 1.02	96.29 ± 4.95

Table 1. Morphometric averages (mean \pm SE) of *Cherax quadricarinatus, Procambarus clarkii* and *Pota-monautes perlatus* used in the macrophyte consumption and fish scavenging experiments.

and Symoens 2005). In the lab, plant matter was rinsed thoroughly under tap water to remove any attached macroinvertebrates. To attain a reliable biomass measurement of the macrophytes, a wet – dry conversion equation was determined by drying known mass of *P. nodosus* (5, 10, 15, 20, 25. 30, 35, 40, 45 and 50 g; n = 3) in an oven at 60 °C for 24 hrs (Madsen and Bloomfield 1993; Bickel and Perrett 2015). The subsequent equation was derived, where dry mass = -0.0043 + 0.1134·wet weight (Suppl. material 1a) (Bickel and Perrett 2015).

Prior to experimentation, the pondweed was patted dry with a paper towel and weighed, then an average of 45.65 ± 0.27 g (equivalent to 5.13 ± 0.03 g dry mass) was put into each experimental tank with an animal. These animals were randomly selected from the holding tanks and patted dry before morphometric measurements were taken for each individual (Table 1). The animals were acclimatised to the experimental tanks for one hour and deprived of food for 24 hrs before the pondweed was added. The experiments were run under a 12:12 light:dark regime for 24 hrs. After the experiment, the remaining macrophytes were patted dry, weighed and dried in an oven to determine the dry weight. Control experiments were run at each temperature treatment with *P. nodosus*, but no consumers.

Fish consumption

Dead O. mossambicus (160.65 ± 1.26 mm, mean total length \pm SE, 74.54 ± 1.59 g mean mass \pm SE) were purchased from Aquaculture Innovations in Makhanda. Experimental fish were kept frozen and defrosted prior to experimentation. Oreochromis mossambicus is native to eastward flowing rivers of central and southern Africa (Skelton 2001). The fish species, together with other Oreochromis species, are commonly referred to as "breams" in the Zambezi Basin and comprise more than 50% of their catch (Tran et al. 2019). Pre-experimental treatment of animals was identical to the macrophyte experiment.

Fish were patted dry and the total length and mass for each fish was recorded. A 50 g sinker was then inserted in their guts through the mouth so that the fish sank to the bottom. The fish were then introduced to the tanks with a consumer in each tank. Controls were also run, where the dead fish were not subjected to any consumer in the experimental tank. Fishermen in the Zambezi system deploy their gillnets around 1600 hrs and retrieve them around 0600 hrs (pers. obs.).

Feeding rates of the three focal species vary with light regime (Madzivanzira et al. 2021a); therefore, to mimic natural conditions these experiments were run in dark from 1600 hrs and terminated at 0700 hrs (i.e. 15 h). At the end of the experiment, crayfish were removed and placed in respective holding tanks. The remains of the fish were removed from the water and placed in a tray with blotting paper for excess water to drip out. The sinkers were removed from the fish. The fish were patted dry and the mass was recorded as well as the parts that were eaten. The parts of fish damaged by the decapods were expressed as the proportion (%) of fish with damage 'i' where 'i' is the area (mouth, eyes, abdomen, fin, gut) damaged by the predator. As it was possible that one fish had several parts damaged, a single fish could have multiple damage categories.

Data analysis

There were morphometric differences between the three species (see Suppl. material 1b), but as consumption was determined per gram of consumer this does not affect the inferences. As we used dry mass as a benchmark to gauge the accuracy of macro-phyte wet mass measurements, dry mass values were used for all macrophyte associated analyses.

In order to compare consumption rates between species and allow data to be relevant to field data, with respect to trends in biomass and individual size varying with time since invasion (Madzivanzira et al. 2021c), we calculated mass of resource consumed per gram of decapod per hour (mass⁻¹ g⁻¹ h⁻¹) (1):

$$Mass^{-1} \cdot g^{-1} \cdot h^{-1} = (N_e / Mass) / T$$
(1)

where N_e is the dry/wet weight of resource; Mass is the mass of individual; and T is the total experimental duration.

A t-test was used determine the extent of natural loss in mass of resource before and after the experiment in the absence of a consumer for the control treatments. As resources were presented separately and dry mass of plant matter used compared to wet mass of fish, two separate generalised linear models (GLM) were used to assess resource consumption. Both GLMs used temperature and species as factors with full interaction terms. Differences between factor levels were assessed using linear contrasts and Tukey HSD.

Differences in parts of fish damaged by the consumers was analysed with 3×7 contingency tables and differences tested with a Chi-square test.

For both resources, the max consumption per g of predator were chosen as the most informative measure, as the respective parameters from functional response analysis are somewhat less meaningful, and this allowed for quantification of the maximum feeding rate per g of predator. The mean mass of each crayfish (Kafue River: 63.22 ± 2.05 g: Lake Kariba: 55.85 ± 1.43 g; Barotse floodplain: 37.18 ± 2.17 g) (Madzivanzira et al. 2021c) and the maximum scavenging rate per gram of *C. quadricarinatus* in 15 h (the number of hours gillnets are deployed) was used to estimate the potential economic losses in catch in the invaded regions of the Zambezi Basin for Kafue River, Lake Kariba and Barotse floodplain. The following equations were used to calculate the economic losses due to crayfish:

$$loss per day (15 hrs) = crayfish consumption (15 hrs) \times crayfish mean mass$$
(2)

monetary loss per day = loss per day × US\$ 1.30 (price of fish per kg)
$$(3)$$

monetary loss per year = monetary loss per day
$$\times$$
 365 (4)

The calculations were done for the low and high temperature treatments which corresponds to the low and high water flow seasons in the invaded regions, respectively.

Results

There was no significant change in resource mass (P > 0.05) from before to after each control experiment, at either of the temperatures; therefore, all change in resource mass is attributed to consumption.

Macrophytes consumption experiment

Temperature and species interacted significantly on the consumption rate of *P. nodo*sus (Table 2), whereby consumption of all the three species was significantly higher at 28 °C than at 19 °C (P < 0.001) (Table 3). Voracity of *P. clarkii* on *P. nodosus* was significantly higher (P < 0.05) than that for *C. quadricarinatus* and *P. perlatus* at both temperatures (Fig. 1), but there was no significant difference between *C. quadricarinatus* and *P. perlatus* (P > 0.05).

Fish scavenging experiment

There was a significant interaction between species and temperature on consumption of *O. mossambicus* (Table 2), whereby increased temperature significantly increased

Table 2. Model terms for all factors from GLM with a quasi-Poisson error distribution used to determine differences in macrophytes consumption and fish scavenging with regards to factors "temperature" and "species", using a Type 3 ANOVA and χ^2 to report the effects.

Model term	Resource	Chi-square	df	P-value
Temperature	P. nodosus	64.64	1	< 0.001
Species	P. nodosus	37.57	2	< 0.001
Temperature × Species	P. nodosus	79.37	1	< 0.001
Temperature	O. mossambicus	85.11	1	< 0.001
Species	O. mossambicus	114.42	2	< 0.001
Temperature × Species	O. mossambicus	143.18	1	< 0.001

Table 3. Mean (±SE) consumption of macrophyte *Potamogeton nodosus* (in 24 hrs) and scavenging of fish *Oreochromis mossambicus* by *Cherax quadricarinatus*, *Procambarus clarkii* and *Potamonautes perlatus* at 19 °C and 28 °C.

Species	Temperature (°C)	Macrophyte Wet mass	Macrophyte Dry mass	Fish scavenged (g)	
		consumed (g)	consumed (g)		
Cherax quadricarinatus	19	4.88 ± 0.62	0.55 ± 0.07	10.50 ± 0.66	
Procambarus clarkii	19	7.29 ± 0.41	0.82 ± 0.05	6.92 ± 0.62	
Potamonautes perlatus	19	3.59 ± 0.59	0.40 ± 0.07	7.59 ± 0.88	
Cherax quadricarinatus	28	9.08 ± 0.62	1.02 ± 0.07	16.77 ± 0.66	
Procambarus clarkii	28	11.48 ± 0.41	1.29 ± 0.05	12.89 ± 0.75	
Potamonautes perlatus	28	7.79 ± 0.59	0.87 ± 0.07	13.89 ± 0.88	



Figure 1. Mean consumption of macrophyte (*Potamogeton nodosus*) per hour per gram of *Cherax quadricarinatus*, *Procambarus clarkii* and *Potamonautes perlatus* at 19 °C and 28 °C. Points indicate raw data values, boxplots indicate ± Standard Error and solid line across the box represents the mean.

consumption of all three species (P < 0.001). Voracity of *C. quadricarinatus* was significantly higher (all P < 0.05) than that for *P. clarkii* and *P. perlatus* at either temperature (Fig. 2), but there was no difference between *P. clarkii* and *P. perlatus* voracity (P > 0.05).

All three decapods caused aesthetic damage to the fish through consumption (See Suppl. material 2). Each scavenger caused significantly different damage to different areas of *O. mossambicus* ($\chi^2 = 152.68$, df = 12, P < 0.001). The two crayfish species

Species	Temperature (°C)	Tail	Abdomen	Fin	Guts	Mouth	Head	Eyes
Cherax quadricarinatus	19	20	19	19	0	0	0	1
Procambarus clarkii	19	20	20	20	1	0	0	0
Potamonautes perlatus	19	1	4	0	0	20	20	20
Cherax quadricarinatus	28	20	17	17	3	4	0	1
Procambarus clarkii	28	20	20	20	1	0	0	0
Potamonautes perlatus	28	0	4	0	1	20	20	20

Table 4. Proportion of fish with different categories of damage *i*.



Figure 2. Mean consumption of fish (*Oreochromis mossambicus*) per hour per gram of *Cherax quadricarinatus*, *Procambarus clarkii* and *Potamonautes perlatus* at 19 °C and 28 °C. Points indicate raw data values, boxplots indicate ± Standard Error and solid line across the box represents the mean.

mostly damaged the tail, abdomen and the fins (proportion > 80%), whilst *P. perlatus* only targeted the head (proportion = 100%) (Table 4).

Potential economic losses

The potential loss in catch due to crayfish scavenging in the invasion cores per fishing night per individual crayfish ranges between: 0.01 - 0.02; 0.01 - 0.02; and 0.01 - 0.01 (Suppl. material 1). This translates to an average annual loss of 6.15; 5.42; and 3.62 per crayfish for Kafue River, Lake Kariba and Barotse floodplain, respectively (Suppl. material 1).

Discussion

High consumption of native resources, relative to that of a native analogue, is regarded as indicative of high impact IAS according to the Resource Consumption Hypothesis (Ricciardi et al. 2013; Paterson et al. 2015; Dick et al. 2017; Laverty et al. 2017). Understanding these impacts on specific ecosystem services is necessary, not only for the regulation and management of these IAS, but also to guard against detriment to human well-being, especially important in areas where food security and water resources are already precarious (Egoh et al. 2020). Here, we compare temperature- and resource-specific feeding rates by invasive crayfish and a native freshwater crab to infer ecological and potential economic impacts on fisheries. We found that consumption of static resources increases with temperature regardless of species or resource and rejected Hypothesis 1. Hypotheses 2 and 3 were also partially rejected due to species specific differences in consumption. Cherax quadricarinatus had a higher impact on dead fish regardless of temperature than the other two species (2) and the same trend was seen in the macrophyte experiment, but in this case, P. clarkii was the most damaging regardless of temperature, thus emphasising the importance of context specific impact assessments to avoid the ambiguity which arises when generalising impacts across families in the absence of species specific evidence per EICAT recommendations (Hawkins et al. 2015). The results also provide maximum feeding rates for the three decapods under two temperature treatments which can be used along with fisheries data in the future to derive potential for economic loss as well as parameterising models.

The temperature treatments in this study directly reflect the conditions in invaded African systems; however, these data can be used globally to gauge temperature-dependent impacts in other areas. Global annual mean temperatures are projected to increase by 1.5 °C between 2030 and 2052 (IPCC 2018). Thus impact of crayfish species will likely increase with the projected climatic changes, as demonstrated in this study. However, the mechanisms and outcomes of ecological impact differ depending on the crayfish species, resource type as well as native analogue dynamics as illustrated by the change in impact patterns between the present study and Madzivanzira et al. (2021a).

All species consumed *P. nodosus* and increased consumption with increasing temperature in line with the metabolic theory of ecology (Brown et al. 2004; Uiterwaal and DeLong 2020). Impact of *C. quadricarinatus* on macrophytes did not differ from that of *P. perlatus*, but *P. clarkii* showed potential for adverse ecological impacts as intense herbivory can have cascading effects across different trophic levels (Marshall 2019). The destruction of macrophytes can also modify nutrient cycling, as a result of removing the stabilising effect of macrophytes upon littoral sediments (Gherardi et al. 2011). *Procambarus clar*kii is well known for high consumption of macrophytes on a global scale (Lodge et al. 2012; Twardochleb et al. 2013; Madzivanzira et al. 2020) and exhibits a preference for plant matter over animal protein (Gherardi and Barbaresi 2007). In Lake Naivasha, the introduction of *P. clarkii* coincided with notable declines in the water lily *Nymphaea nouchalii var. caerulea* suggesting consumptive impacts on this macrophyte (Lowery and Mendes 1977). This high preference for macrophytes by *P. clarkii* explains the difference between the comparatively low impact on juvenile fish prey in Madzivanzira et al. (2021a) and the high impact in the macrophyte experiment of the present study. The high consumption of macrophytes by *P. clarkii* could be related to feeding and processing morphology as *P. clarkii* has thin chelae and a low closing force (South et al. 2020) and a gastric mill which may specialise them for processing plant matter over other resources (Chisaka and Kozawa 2003; McGaw and Curtis 2013). *Cherax quadricarinatus* is an emerging invader with few recorded impacts (Haubrock et al. 2021). However, introductions into the Pilbara Region of Australia resulted in the complete loss of macrophyte cover and subsequent community reorganisation (Pinder et al. 2019) and, in Lake Kariba, Zimbabwe, macrophytes dominated the diet of *C. quadricarinatus* across size ranges (Marufu et al. 2018).

All three species showed propensity for scavenging behaviour on dead fish, corroborating the anecdotal accounts of crayfish destruction of fisher catch (Weyl et al. 2017; Madzivanzira et al. 2020). Cherax quadricarinatus consumption was more pronounced in the fish scavenging experiment, to the extent that consumption at the lowest temperature was still higher than that of *P. perlatus* at the highest temperature. The results are similar to Madzivanzira et al. (2021a) in that C. quadricarinatus had the highest impact on fish resources; however, *P. perlatus* did not suffer from a reduction in per capita consumption with increased temperature in the present study. This suggests that the results in Madzivanzira et al. (2021a) are likely due to a temperature driven mismatch in attack and escape speeds of P. perlatus and Clarias gariepinus, rather than the physiological performance of *P. perlatus* under high temperature. In contrast, *P.* clarkii had similar scavenging rates to P. perlatus, indicating a possible lack of impact on fish catch. However, aesthetic damage to catch often translates to economic loss regardless of extent. The two crayfish species damaged mostly the posterior parts of the fish, whilst the crabs damaged mostly the anterior parts. The fish head, preferentially damaged by the crabs, contains higher nutrient content compared to other body parts of the fish (Petricorena 2014). The higher closing force of crab chela compared to the two crayfish species may facilitate access to the anterior parts (head) of the fish which are tougher compared to the soft parts (abdomen and guts) which were more likely to be damaged by the crayfish species (South et al. 2020).

Both resource types investigated here have direct and indirect economic implications besides the ecological ramifications of generalist omnivores on aquatic communities. Healthy and high integrity macrophyte stands provide crucial fish nursery habitat and indirectly support fishery productivity and resilience (Choi and Kim 2020). The loss of macrophyte beds in Kenya due to *P. clarkii* invasion reduced food resources for a variety of African wetland birds (Taylor and Harper 1988; Harper et al. 2002) which indirectly negatively affects ornithological tourism (Gherardi et al. 2011). Inland fisheries provide livelihoods and ecosystem services for millions of people globally (Lynch et al. 2020). African artisanal fisheries suffer from pressures similar to most capture fisheries worldwide, for example, overexploitation, unemployment and rapid population growth (Tweddle et al. 2015). Fish products form part of a larger value chain commercially and when crayfish cause a percentage of the catch to be unmarketable as a result of scavenging, targets are not met and the impacts cascade to the public, making the situation a food security cause for concern. The impact is aggrandised by low overall fish catches as crayfish entangle themselves in the gillnets, thereby reducing the efficiency of these gillnets (Weyl et al. 2017) and, further, as fishers must then increase their fishing effort to compensate for the lost catch. These dynamics might not be isolated to African systems alone (see Madzivanzira et al. 2020) and should not be underestimated. In Europe, crayfish have been shown to cause serious damage to carp rigs by clawing and nipping at the line and scavenging on bait for catching fish (see https://carp-fishing-reels.com/blog/general-advice/combatting-crayfish/). The artisanal fishery is likely to be further threatened by low catches as the crayfish species were shown to be able to consume a high number of catfish fry (Madzivanzira et al. 2021a) which could affect recruitment, productivity/yield and hence human livelihoods.

This study also estimated the potential monetary losses fishermen are likely to experience due to catch spoilage by crayfish in the invaded regions of the Zambezi Basin. The study showed high potential economic impacts in older invasions (Kafue and Lake Kariba). The potential losses in catch and income shown in this study could be even greater in the field, because the mass consumed in the lab was used to up-calculate the overall mass lost due to crayfish spoilage. This overall mass may under-represent the spoiled catch as when crayfish consume a small amount/part of the fish in the field, the whole fish is regarded as spoiled. Over- and underestimation of the losses can result in several assumptions such as that crayfish feed only on fish caught in the gillnets (overestimation in this case), not considering that small amounts consumed ruin the entire fish for sale (underestimation) and not considering fishing bans (overestimation). While this study gives a snapshot of the potential losses due to crayfish invasions, field surveys and further investigations are more appropriate to calculate the realistic losses in catch and income.

Incorporating context-specific comparisons with an ecologically relevant native trophic analogue is essential to determine the relative difference in resource consumption (Dick et al. 2017). The results of the present study show that, on a 1:1 (g) basis, the impact of both invasive crayfish is comparable to P. perlatus which seems to more provide evidence for possible biotic resistance (see South et al. 2020). Nonetheless, freshwater crabs, while ubiquitous across the continent, are relatively low in abundance and suffer from large data deficits in basic ecology which can confound comparative inferences (Madzivanzira et al. 2020; South et al. unpublished data). The invasion by crayfish species can lead to more diverse impacts and threaten resources that were not previously threatened by the crabs alone. We stress the need to combine laboratory data, such as the present study and Madzivanzira et al. (2021a) with contextually relevant field abundance patterns to improve prediction of impact magnitude (Dick et al. 2017; Zeng et al. 2019; Dickey et al. 2020). It is, thus, likely that the actual field impact of crayfish invasions is exacerbated by extreme differences in relative abundance between trophic analogues (South et al. 2020; Madzivanzira et al. 2021c, South et al. unpublished data). The derivation of temperature-specific per gram maximum feeding estimate for global invaders can facilitate rapid assessments and comparisons from other invasion cores which ultimately will assist in hypothesis testing and impact prediction.
Crayfish invasions have high negative implications for ecology and socio-economic dynamics of the recipient area. Intersectional adverse impacts are likely to persist and escalate, especially considering the low level of conservation management resources available (Madzivanzira et al. 2020). The pressing issue of unhindered crayfish invasions, especially in Africa, needs to be prioritised as the food security of livelihoods in invaded regions will be affected. There is need to investigate whether results from this study translate to the actual declines in catches through fish catch assessments and value chain analysis, while considering field abundance patterns. However, this relies upon interdisciplinary collaboration to compile the relevant information for robust assessment.

Data availability statement

The raw data generated and used in the analysis, as well as the data that supports the use of the temperature treatments, are publicly available at: https://doi.org/10.6084/m9.figshare.15019593.v2.

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Supplementary material I

Macrophyte dry weight determination and morphometric averages (\pm SE) of used animals

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Data type: regression analysis and morphometric measurements

- Explanation note: The supplementary file shows the graph that was used for the wet dry macrophyte weight conversion. The file also shows the mean mophometric measurements of the decapods and reports the statistics to determine their differences.
- Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Link: https://doi.org/10.3897/neobiota.72.71868.suppl1

Supplementary material 2

Field and laboratory photos showing crayfish damage

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Data type: images

- Explanation note: Supplentary file 2 shows photos of field and lab-based evidence of crayfish impacts on the artisanal fishery as reported in Southern Africa.
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What is valued in conservation? A framework to compare ethical perspectives

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Abstract

Perspectives in conservation are based on a variety of value systems. Such differences in how people value nature and its components lead to different evaluations of the morality of conservation goals and approaches, and often underlie disagreements in the formulation and implementation of environmental management policies. Specifically, whether a conservation action (e.g. killing feral cats to reduce predation on bird species threatened with extinction) is viewed as appropriate or not can vary among people with different value systems. Here, we present a conceptual, mathematical framework intended as a tool to systematically explore and clarify core value statements in conservation approaches. Its purpose is to highlight how fundamental differences between these value systems can lead to different prioritizations of available management options and offer a common ground for discourse. The proposed equations decompose the question underlying many controversies around management decisions would likely be viewed under three idealised value systems: ecocentric conservation, which aims to preserve biodiversity; new conservation, which considers that biodiversity can only be preserved if it benefits humans; and sentien-

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tist conservation, which aims at minimising suffering for sentient beings. We illustrate the utility of the framework by applying it to case studies involving invasive alien species, rewilding, and trophy hunting. By making value systems and their consequences in practice explicit, the framework facilitates debates on contested conservation issues, and complements philosophical discursive approaches about moral reasoning. We believe dissecting the core value statements on which conservation decisions are based will provide an additional tool to understand and address conservation conflicts.

Keywords

Anthropocentrism, biocentrism, ecocentrism, environmental ethics, impact, invasive alien species, moral values, sentientism, speciesism

Introduction

The consideration of the moral relationship between people and nature and the consequent ethical obligations for conservation is relatively recent in Western culture. Environmental ethics emerged as an academic discipline in the 1970s (Brennan and Lo 2016) and the concepts of values, duty, and animal welfare, are increasingly appreciated in applied ecology and conservation (Dubois et al. 2017; Díaz et al. 2018). These concepts are complex, and the formulation and implementation of environmental management policies is often associated with conflicts between different groups of stakeholders and between people with different values and interests, for example for the management of charismatic alien species (Redpath et al. 2013; Crowley et al. 2017; Jarić et al. 2020). An examination of how value systems could be explicitly accounted for in conservation decisions could offer opportunities for better identifying conflicts, potentially helping to resolve them, and overall improve environmental management.

Value systems consider more or less inclusive communities of moral patients, defined as the elements with intrinsic or inherent value towards which humans, considered here as the community of moral agents, are considered to have obligations (in the following, for simplicity, we refer to the community of moral patients as the moral community; Table 1). Moral communities can include only humans (anthropocentrism), to further incorporate sentient beings (sentientism), living beings (biocentrism), and collectives (such as species and ecosystems; ecocentrism) (Table 1, Fig. 1). The definition of moral communities can also be influenced by additional elements (such as spatial elements in the case of nativism), and, at the assessor level, by personal experience. These value systems underlie different sets of explicit or implicit normative postulates, i.e. value statements that make up the basis of an ethic of appropriate attitudes toward other forms of life, which, in turn, can form the basis of different conservation approaches (Soulé 1985; Table 1). If the normative postulates of different value systems diverge (and excluding considerations that moral reasoning, experience, etc., may change one's value system), conflicts can arise between different groups of stakeholders whose members share common moral values (Crowley et al. 2017). In particular, conservationists who value biodiversity per se [as defined initially by Soulé

Term	Definition
Anthropocentrism	Value system that considers humans to be the sole, or primary, holder of moral standing, and therefore the
(strong)	concern of direct moral obligations. Non-human species are considered only to the extent that they affect the
0	satisfaction of felt preference of human individuals (Norton 1984; Rolston 2003; Palmer et al. 2014).
Anthropocentrism	Value theory in which all values are "explained by reference to satisfaction of some felt preference of a human
(weak)	individual or by reference to its bearing upon the ideals which exist as elements in a world view essential to
	determinations of considered preferences" (Norton 1984). That is, the value of an individual or species is not only
	exploitative, but incorporates human experience and the non-utilitarian relationship between humans and nature.
Anthropomorphism	"The attribution of human personality or characteristics to something non-human, as an animal, object, etc."
	(Oxford English Dictionary 2021a).
Biocentrism	Value system considering all living beings as the concern of direct moral obligations (Rolston 2003; Palmer et al. 2014).
Collectivism	Value system in which a group or collective has a higher value than the individuals that compose it (Wallach et
	al. 2018).
Compassionate	Conservation approach inspired by virtue ethics based on four tenets: i) do no harm; ii) individuals matter; iii)
conservation	inclusivity (the value of an individual is independent from the context of the population, e.g. nativity, rarity,
	etc.); and iv) peaceful coexistence (Ramp and Bekoff 2015; Wallach et al. 2018).
Community of	The group of beings considered to have moral responsibility in their actions (Talbert 2019). We consider it
moral agents	here to be restricted to humans.
Community of	The group of beings considered to have intrinsic moral value, and towards which moral agents have moral
moral patients	obligations (warren 2000). The size of the group (referred to as the moral community in this work, for
	of Anthropocentriem
Conservation	Conservation approach aiming at minimizing animal suffering (Beausaleil et al. 2018)
welfare	Conservation approach anning at minimizing annina sunering (beausoich et al. 2010).
Consequentialism	"An ethical doctrine which holds that the morality of an action is to be judged solely by its consequences"
-	(Oxford English Dictionary 2021b).
Convergence	"If the interests of the human species interpenetrate those of the living Earth, then it follows that
hypothesis	anthropocentric and non-anthropocentric policies will converge in the indefinite future" (Norton 1986).
Deontology	A normative ethical theory considering that "choices are morally required, forbidden, or permitted" (Alexander
	and Moore 2016).
Ecocentrism	Value system considering that species, their assemblages and their functions, as well as more broadly
	ecosystems, rather than individuals, are the concern of direct moral obligations (Rolston 2003; Palmer et al. 2014).
Empathy	"The quality or power of projecting one's personality into or mentally identifying oneself with an object of
1 /	contemplation, and so fully understanding or appreciating it." (Oxford English Dictionary 2021c). Empathy
	will influence the inherent value given to individuals from other species.
Impact (for the	Impact refers to any effect that modifies the wellbeing, health or resilience (for non-sentient beings) of an
purposes of the	individual, from physical pain to emotional suffering and death (these notions being interrelated, but not
framework, Eq.1)	equivalent).
Inherent value (our	Value possessed by an individual or collective, accounting for their intrinsic value (see definition below) and
definition)	the effects of multiple context-dependent factors (e.g. charisma, anthropomorphism, organismic complexity,
	neoteny, cultural importance, religion, or parochialism). For example, wolves and dogs may be considered to
	have similar intrinsic value under sentientism because they have similar cognitive abilities, but may be valued
T 1	All the second does a spets (i.e. due to parochiaism).
Intrinsic value	value possessed by an individual or collective as defined by a system of moral valuation, such as
	with the system of values (e.g. cognitive ability under sentientism, the choice of a criterion itself may be
	subjective), intrinsic value is determined by this criterion and does not vary with the context (cf. inherent value).
Invasive alien species	"Plants, animals, pathogens and other organisms that are non-native to an ecosystem, and which may cause
	economic or environmental harm or adversely affect human health" (<i>Regulation (EU) No 1143/2014 of the</i>
	European Parliament and of the Council of 22 October 2014 on the prevention and management of the introduction
	and spread of invasive alien species).
Moral community	See "Community of moral patients" and cf. "Community of moral agents".
Moral dilemma	Situation in which a moral agent regards itself as having moral reasons to do different, incompatible actions (McConnell 2018).
Nativism	Value system considering that species that have evolved in a given location have a higher value in this location
	than species that have evolved somewhere else. In nativism, value varies spatially (Wallach et al. 2018).

Table I. Glo	ossary of terms	as they are	used for the	purposes of th	his paper.
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Term	Definition			
Nature despite	Management conceptual approach aiming at conserving biological diversity (focusing on species and habitats)			
people	specifically in response to human impacts on the environment, e.g. sustainable use (Mace 2014).			
Nature for itself	Management conceptual approach aiming at conserving biological diversity (focusing on wilderness and natural			
	habitats) through human exclusion, for example through the creation of parks and protected areas (Mace 2014).			
Nature for people	Management conceptual approach aiming at conserving the components of nature beneficial to humans			
	(focusing on ecosystems and their services) (Mace 2014).			
Neoteny	"The retention of juvenile characteristics in a (sexually) mature organism" (Oxford English Dictionary 2021d).			
New conservation	Discipline aiming at preserving biological diversity through the conservation of natural elements providing			
	services and contribution to human wellbeing (Kareiva and Marvier 2012; Kareiva 2014).			
Normative postulate	Value statements that make up the basis of an ethic of appropriate attitudes toward other forms of life (Soulé 1985).			
Parochialism	Ideology in which moral regard is directed "towards socially closer and structurally tighter targets, relative to			
	socially more distant and structurally looser targets", and, by extension, to species phylogenetically, cognitively,			
	or in appearance closer to humans (Waytz et al. 2019).			
People and nature	Management conceptual approach considering that humans and nature are interdependent and therefore			
	aiming at achieving compromises in the conservation of nature and human wellbeing (Mace 2014).			
Relational value	"Preferences, principles, and virtues associated with relationships, both interpersonal and as articulated by			
	policies and social norms [] Relational values are not present in things but derivative of relationships and			
	responsibilities to them." (Chan et al. 2016).			
Sentience	The ability to experience phenomenal consciousness, i.e. the qualitative, subjective, experiential, or			
	phenomenological aspects of conscious experience, rather than just the experience of pain and pleasure (Allen			
	and Trestman 2017).			
Sentientism	Value system considering sentient beings as the concern of direct moral obligations (Rolston 2003; Palmer et			
	al. 2014).			
Speciesism	Value system in which some species are considered to have a higher value than others, for various possible			
	reasons (Singer 2009). Speciesism is often used to refer to the superiority of humans, which is a specific			
	expression of speciesism as considered in this paper.			
Suffering	Negative emotion, sometimes called emotional distress, experienced by sentient beings, and which can result			
	from different causes, including but not limited to physical pain (Dawkins 2008; Farah 2008).			
Traditional	Discipline aiming at preserving biological diversity through the management of nature, and based on four value-			
conservation	driven normative postulates: "diversity of organisms is good," "ecological complexity is good," "evolution is			
	good," and "biotic diversity has intrinsic value" (Soulé 1985). Traditional conservation is rooted in ecocentrism.			
Utilitarian value	Value given to an individual or collective by humans, based on its utility. For example, dogs may have a			
	utilitarian value for herding sheep or as guard-dogs (see also inherent value). In our framework, utilitarian			
	value is expressed through the impact I on the species with inherent value (i.e. the moral community), but is			
	not expressed explicitly through V (Eq. 1).			
Virtue ethics	Ethical doctrine that emphasises the virtues, or moral character as the reason for action (Hursthouse and			
	Pettigrove 2018).			

(1985), called hereafter 'traditional conservation' (Table 1)] can be at odds with those who value biodiversity based on human welfare and economic aspects [including 'new conservation' (Kareiva and Marvier 2012)], or with those based on animal welfare ['conservation welfare' (Beausoleil et al. 2018), or, to a certain extent, 'compassionate conservation' (Wallach et al. 2018)]. These issues have been heatedly debated in the literature (Kareiva 2014; Soulé 2014; Doak et al. 2015; Driscoll and Watson 2019; Hayward et al. 2019).

In the following, our aim is to conceptualise and decompose value systems in an explicit, and potentially (but not necessarily) quantifiable, fashion using a common mathematical framework, and to explore their repercussions for the perception of conservation management actions by stakeholders with different value systems. We argue that doing so allows for explicit comparison between these perceptions to identify sources of potential conflicts. First, we recapitulate four archetypal value systems in environmental affairs and relate them to different conservation philosophies. Since



Figure 1. Differences between the moral communities considered by value systems influenced by anthropocentrism, sentientism, biocentrism and ecocentrism (depicted by the nested circles and colours) and how values can differ between members of the different moral communities. a) Anthropocentrism, sentientism and biocentrism all value individuals intrinsically, but consider different moral communities, i.e. the value of an individual depends on the category of species it belongs to, with {humans} \in {sentient beings \in {all living organisms}. Species outside of the moral community may have a utilitarian value for species in the moral community (represented by the arrow), which will be reflected by changes in the impact variable. b) The intrinsic value, in combination with contextual factors, defines the inherent value Vof an individual or species and the distribution of V will change depending on the set of species included in the moral community. Anthropocentrism, sentientism and biocentrism value individuals from different groups of species. Biocentrism and ecocentrism give value to the same group of species, i.e. all living organisms, but while biocentrism values individuals, ecocentrism values ecological collectives, i.e. species or species assemblages and ecosystems. Note that species can have both an inherent and a utilitarian value. Within the moral community, species may have equal inherent values, but subjective perceptions and different value systems may also assign different values to different species. The skewness of the value distribution then indicates the degree or strength of speciesism with respect to the species of reference, assumed here to be the human species, and is influenced by many factors, including charisma, cultural context, etc.

identifying commonalities in the perspectives of different parties is key in conflict management (Redpath et al. 2013), we then introduce a formal framework to conceptualise these value systems, and examine how it can be applied to clarify different perspectives. Finally, we discuss opportunities for identifying commonalities between different value systems that may help to identify widely acceptable solutions to otherwise polarising issues.

Value systems and conservation practices

Here, we focus on a Western perspective of value systems that have been internationally considered for environmental policies and the management of nature (Mace 2014). The archetypes of value systems and of conservation approaches were chosen for their importance in the past and present literature and their clear differences, to illustrate our framework. We acknowledge this is a small part of the global diversity of value systems. It would be interesting to see if our framework could be applied to other contexts, to identify its limitations.

From the valuation of humans to that of ecosystems: a spectrum of value systems in conservation

The Western perspective of moral valuation encompasses a diverse set of value systems with respect to the components of nature that form the moral community. Traditionally, one can distinguish at least four archetypal value systems: anthropocentrism, sentientism, biocentrism, and ecocentrism (Rolston 2003; Palmer et al. 2014) (Table 1; Fig. 1).

Anthropocentrism values nature by the benefits it brings to people through ecosystem services, which encompasses economic, biological, and cultural benefits humans can derive from nature (Díaz et al. 2018). One justification for anthropocentrism is that humans are (arguably) the only self-reflective moral beings, and people are both the subject and object of ethics (Rolston 2003), therefore constituting the moral community. In an anthropocentric system, individuals from non-human species only have value based on their benefits or disservices for humans (instrumental).

Sentientism considers that humans and all sentient animals value their life, and experience pleasure, pain, and suffering (Table 1). All sentient individuals should therefore also be part of the moral community (i.e. have an intrinsic value). In this view, it is the sentience [e.g. measured through cognitive ability, (Singer 2009)], rather than species themselves, that has intrinsic value.

Biocentrism considers that life has intrinsic value. Although different perspectives on why life has value exist (Taylor 2011), all living organisms are valued equally for being alive, and not differently based on any other trait.

Some ecocentric, or holistic, value systems consider that ecological collectives, such as species or ecosystems, have intrinsic value, independently from the individuals that comprise them. Species can have different values, i.e. speciesism (Table 1), and these values can be influenced by a multitude of factors, discussed in more detail below.

Subjective elements in the valuation of nature

In practice, the separation between anthropocentrism, sentientism, biocentrism, and ecocentrism is blurry, and values given to different species may vary under the same general approach. For example, biocentrism can range from complete

egalitarianism between organisms to a gradual valuation resembling sentientism. These four value systems can also interact with other systems that use other criteria than the intrinsic characteristics of individuals to define the moral community. For example, nativism is a system that values organisms indigenous to a spatial location or an ecosystem over those that have been introduced by humans. Nativism can therefore interact with any of the four systems presented above to alter the value attributed to a species in a given context. Finally, how someone values individuals of different species is often influenced by their personal views and experiences (Palmer et al. 2014; Waytz et al. 2019). Values and personal interests thus interact when people make and express moral judgements (Essl et al. 2017). Therefore, the archetypes of value systems presented above are rarely expressed in a clear and obvious fashion. Nonetheless, by formalising the archetypes, a framework can be created within which the consequence of conservation actions can be explored (see 'consequentialism' vs. 'deontology', Table 1).

To account for the different elements that can be combined to create the concept of value, in the following, we distinguish between 'intrinsic', 'inherent', and 'utilitarian', value (our definitions; Table 1). Intrinsic value is the value possessed by an individual or collective as defined by one of the systems above, and is therefore independent of context. Intrinsic value is based on objective criteria such as cognitive ability. The choice of a criteria may be subjective, but the value is independent of the assessor once the criteria has been defined. This has been termed "objective intrinsic value" by others (Sandler 2012). Inherent value is the value of an individual, species or ecosystem that results from the combination of its intrinsic value and context-specific and subjective factors (note that other scholars have used 'inherent' differently, e.g. (Taylor 1987; Regan 2004); here it corresponds to what has also been termed "subjective intrinsic value" (Sandler 2012)). These factors include charisma (Courchamp et al. 2018; Jarić et al. 2020), anthropomorphism (Tam et al. 2013; Table 1), organismic complexity (Proença et al. 2008), neoteny (Stokes 2007; Table 1), cultural importance (Garibaldi and Turner 2004), religion (Bhagwat et al. 2011), parochialism (Waytz et al. 2019; Table 1), and more generally the relationship between humans and elements of nature (Chan et al. 2016). For example, dogs and wolves may be considered to have similar cognitive abilities objectively, and therefore a similar intrinsic value under sentientism, but dogs may have a higher inherent value for some people because they are in close contact with individuals from this species, i.e. parochialism. Some alien species that did not have any inherent value prior to their introduction have been incorporated in local cultures, therefore providing them a novel and higher inherent value such as horses being linked to a strong local cultural identity in some parts of the USA (Rikoon 2006). Inherent value can often be considered to be fixed at the time scale of a management action, but can nonetheless vary over short time scales in some situations (see the example of the Oostvaardersplassen nature reserve below). Utilitarian value is determined only from an anthropocentric perspective. It is context-dependent and can change rapidly, for example in the case of commercial exploitation.

Conservation management derived from value systems

Conservation practices can historically be divided into three main categories, closely related to specific systems of moral valuation (Mace 2014). At one extreme, a 'nature for itself' or 'nature despite people' (Table 1) view mostly excludes humans from the assessment of the efficacy of conservation management actions (Fig. 2). This ecocentric perspective is the foundation of traditional conservation as defined by Soulé (1985), and relies on the four following normative postulates: "diversity of organisms is good", "ecological complexity is good", "evolution is good", and "biotic diversity has intrinsic value" (Soulé 1985). It historically underlies widely-used conservation tools, like the IUCN Red List of Threatened Species (IUCN 2019), in which threat categories are defined in terms of probability of extinction (Mace and Lande 1991) (i.e. a species-level criterion aimed at preserving biodiversity). Ecocentrism is often not limited to the valuation of species, but can encompass wider collectives, i.e. assemblages of species and functions, or ecosystems. This other perspective is captured, for example, by the IUCN Red List of Ecosystems (IUCN-CEM 2016), and it is strongly reflected in international conservation agreements such as the Convention on Biological Diversity (UNEP CBD 2010). In the following we refer to traditional conservation as an ecocentric value system where species are intrinsically valuable (nature for itself; Fig. 1, 2) and humans are mostly excluded from management. We acknowledge that this is an archetypal view of traditional conservation, which is used here simply for illustrative purposes.

By contrast the more recent, anthropocentric 'nature for people' perspective (Mace 2014) values species and ecosystems only to the extent that they contribute to the wellbeing of humans (Fig. 2). These values encompass ecosystem services that help sustain human life (Bolund and Hunhammar 1999) or economic assets (Fisher et al. 2008), and can rely on the assessment of species and ecosystem services in terms of their economic value (Costanza et al. 1997), which can be considered as the most general form of utilitarian value, and has also been termed economism (Norton 2000). The 'nature for people' perspective can nonetheless incorporate additional measures linked to human wellbeing, such as poverty alleviation or political participation. This more holistic measure of impacts on humans is exemplified by 'new conservation', also termed 'social conservation' (Miller et al. 2011; Kareiva 2014; Doak et al. 2015) (Table 1; Fig. 2). It has been argued that such an anthropocentric perspective will, by extension, help and even be necessary to maximise the conservation of nature (Kareiva and Marvier 2012). Although new conservation was introduced relatively recently (Fig. 2), it follows an older perspective termed the convergence hypothesis, which argues that if human interests depend on the elements of nature, conservation approaches motivated by anthropogenic instrumental or non-anthropogenic intrinsic values should be the same (Norton 1986; Table 1). It is important to note that the exact set of normative postulates proposed by the proponents of new conservation is not clearly defined (Miller et al. 2011), leading to differences of interpretation and heated debates in recent years (Kareiva and Marvier 2012; Kareiva 2014; Soulé 2014; Doak et al. 2015).



Figure 2. Different value systems (or combination of) correspond to different conservation perspectives, which were introduced at different points in time (the timeline is approximate for illustrative purpose; see also Mace 2014). A nature for itself perspective can be either ecocentric, biocentric, or both. Under new conservation, an anthropocentric perspective is considered necessary to achieve a desirable outcome under a biocentric perspective (\Rightarrow). Under the people and nature approach, anthropocentric, biocentric and ecocentric perspectives are considered simultaneously (+). Underlying concepts and movements predating conservation approaches are indicated in grey italic at the approximate period they originated.

More recently, the necessity to account for the interdependence between the health of nature and human wellbeing [i.e. 'people and nature' (Mace 2014); Fig. 2] has been advocated in the United Nations Sustainable Development Goals (Weitz et al. 2018). This approach lies on the notion of weak anthropocentrism, introduced by the environmental pragmatism movement (Norton 1984; Katz and Light 2013), in which the value of elements of the environment is not only utilitarian, but defined by the relationship between humans and nature (Chan et al. 2016), and therefore is influenced by context and people's experience (see also the notion of inherent value described above). Similarly, "nature-based solutions" is an approach endorsed by the IUCN, which aims at protecting, sustainably managing, and restoring, natural or modified ecosystems, to simultaneously provide human wellbeing and biodiversity benefits (Cohen-Shacham et al. 2016). The 'One Health' approach, endorsed by the Food and Agriculture Organization, the World Health Organization, and the World Organisation for Animal Health also acknowledges the interdependence between the state of ecosystems, human health, and zoonoses (Gibbs 2014). The difference between people and nature and new conservation approaches therefore lies in the fact that it merges anthropocentric and ecocentric systems, rather than considering that the latter will be addressed by focusing on the former (see Section "Nature despite/for/and people" below for details).

Finally, although the animal rights movement, based on sentientism, originated in the 19th century (Salt 1894), it has not, to our knowledge, been formally considered in conservation approaches until recently. Two main approaches can be found in the literature. Conservation welfare (Beausoleil et al. 2018) is a consequentialist perspective that considers conservation under the prism of animal welfare maximisation (Fig. 2). Compassionate conservation (Ramp and Bekoff 2015; Wallach et al. 2018), also incorporates animal sentience, but from a virtue ethics perspective (Table 1). Although conservation welfare aims at aligning with more traditional conservation approaches presented above (Beausoleil et al. 2018), compassionate conservation appears to be set on different values and proposes, for example, to incorporate emotion to provide insight in conservation (Batavia et al. 2021).

Framing moral values for objective-driven conservation

Formulation of a mathematical framework

Many of the conflicts in conservation are grounded in the failure to identify and formalise differences in world views, which contain elements of the four archetypes presented above, influenced by cultural norms, economic incentives etc. (Essl et al. 2017). Here, we propose a mathematical formulation as a method to clarify moral discourses in conservation, based on a consequentialist perspective. We therefore consider an objective-driven type of conservation. Our purpose is not to argue about the relevance of consequentialism vs. deontology, or on the place of virtue ethics in conservation. Rather, we consider that, from a management perspective, conservation necessarily includes objective-driven considerations. A better understanding of how and why objectives can differ between stakeholders as a result of their value systems is therefore useful to anticipate and manage potential conflicts. Although some participants of the discourse will be more receptive to discursive than mathematical conceptualisation, we argue that defining concepts as mathematical terms can make differences in value systems and their normative postulates more explicit and transparent, which will be beneficial when used with appropriate stakeholders, even when these terms would be hard to quantify in real life. A mathematical formulation can be seen as a logical way to express relationships between different elements. Doing so can help to identify and facilitate the discussion of shared values and incompatibilities between different environmental policies and management options (Miller et al. 2011), and contribute to manage conflicts (Redpath et al. 2013). In a similar vein, Parker et al. (1999) proposed a mathematical framework for assessing the environmental impacts of alien species. This work was highly influential in the conceptualisation of biological invasions (being cited over 2,000 times until April 2021 according to Google Scholar), rather than by its direct quantitative application. We also acknowledge that this approach has specific limitations, which are discussed below.

Our mathematical formalisation conceptualises the consequences of environmental management actions. As we develop below, these consequences will be defined differently depending on the value system, but can be understood generally as the consequences for the members of the moral community. Under anthropocentrism, these will be consequences for humans; under sentientism, these will be consequences for sentient individuals; under biocentrism and ecocentrism, these will be consequences for biodiversity. We argue that our mathematical formalisation can account for these different value systems (see Suppl. material 1: Appendix S1 for an extension to ecocentrism beyond species and considering wider collectives, i.e. ecosystems), while also accounting for cultural and personal contexts. These consequences *C* can be conceptualised as a combination of the impact of an action on the different species or individuals involved and the value given to said species and individuals under different value systems as follows:

$$C = \sum_{\text{species } s} \overline{I_s} \times V_s \times N_s^{\ a}$$
 Eq.1

where \bar{I}_s is a function (e.g. mean, maximum, etc.) of the impact (direct and indirect) resulting from the management action on all individuals of species *s*, V_s is the inherent value attributed to an individual of species *s* (as described above), N_s is the abundance of species *s*, and *a* determines the importance given to a species based on its abundance or rarity (and enables to account for the importance of a species rather than an individual, see below). The unit of *C* depends on how other parameters are defined, which themselves depend on the value system considered. In summary, the higher the impact on species with high values, the higher the consequences.

Inherent value V_s can have a monetary unit or be unit-less depending on how it is defined. It can be continuous or categorical (e.g. null, low, high – quantifiable as 0, 1, 2 or any other quantitative scale). Our definition of inherent value here is extremely broad, as the purpose of this work is not to define what such value should be, rather, it is to be flexible enough to encompass multiple perspectives and the subjectivity of the assessor, and be based on intrinsic, utilitarian or relational values (Chan et al. 2016; Table 1).

The parameter *a* can take both positive and negative values. A value of 1 means that consequences are computed over individuals. If all values V_s were the same, a = 1 implies that all individuals in the moral community (Table 1) weigh the same when computing C, irrespective of the species they belong to. This is typical of individual-centred value systems, i.e. sentientism, and biocentrism, whose characteristics (sentience and life) are defined at the individual level. As a result, impacts on larger populations would weigh more on the consequences. As *a* decreases towards 0, the correlation between the value of a species and its abundance decreases. For a = 0, the consequence of a management action becomes abundance-independent. For a < 0, rare species would be valued higher than common species (or the same impact would be considered to be higher for rare species), for example due to the higher risk of extinction. And for a > 1, disproportionate weight is given to abundant species, which are often important for providing ecosystem services (Gaston 2010).

The impact I is computed at the individual level. It can be limited to the probability of death of individuals or changes in per capita recruitment rate, thus allowing to compute a proxy for extinction risk if $a \leq 0$, but can also include animal welfare, biophysical states, etc. As for V, continuous or categorical scales may be used. Different measures of impact can be considered under a same system of value, in which case Equation 1 should be applied to each one separately (see section "Application of the mathematical framework" below for details). I can only encompass the direct impact of a management action (in a narrow view that only the direct impact of humans, i.e. the moral agents, should be considered, and that the direct impacts from non-moral agents should not be considered), but also include its indirect impact resulting from biotic interactions (considering that, in the context of management and therefore human actions, these indirect impacts are ultimately the result of the actions of the moral agents). One would therefore need to define a baseline corresponding to either i) the lowest possible measurable level of impact (e.g. being alive if death is the only measure of impact, or no sign of disease and starvation for biophysical states; this would obviously be more complicated for welfare), so that I would only be positive; ii) the current state of the system, in which case impacts could be positive or negative for different species; or iii) the past state of a system, for example prior to the introduction of alien species (see (Rohwer and Marris 2021) for a discussion on the notion of ecosystem integrity). The duration over which to measure such impact should also be determined. The exact quantification of impact will be influenced by different value systems and personal subjectivity. Some impacts may be considered incommensurable (Essl et al. 2017), therefore falling out of the scope of the framework. The average impact I over all considered individuals from a species could be used as a measure at the species-level, as different individuals may experience different impacts, if the management action targets only part of a given population. Using the average impact is not without shortcomings though, since it does not account for potential discrepancies in impacts suffered by different individuals in a population. In other words, to which point do "the needs of the many outweigh the needs of the few" (Littmann 2016)? Other measures such as the maximum impact experienced by individuals, or more complex functions accounting for the variability of impacts and values across individuals of a same species may also be used, to account for potential disproportionate impacts on a subset of the considered individuals. Under anthropocentric perspectives, impacts are influenced by the utilitarian values of species.

Application of the mathematical framework

Considering Equation 1 in an operational fashion, the consequences *C* computed from it can be interpreted as a constructed attribute to measure the achievement of objectives in conservation under different value systems (sensu Keeney and Gregory 2005). This may be possible for simple systems with few species and clear categories of values and impacts (Fig. 3). However, for complex systems, a quantitative evaluation of Equation 1 will be difficult or impossible. For such systems, the purpose of the framework is



Figure 3. Applying the framework presented in Equation 1 to determine the likely consequence of a management action on a system with two species, highlighting possible moral dilemmas in red. In the case shown *a* is set to 1 for simplicity, but the two species have different inherent values V_{hieh} and V_{low} (i.e. how individuals are valued does not vary with abundance, but individuals of one species are valued more than the other species). The likely consequence changes with the relative abundance of the two species [top row (a) vs. bottom row (**b**)] and with whether the impact of the management intervention is positive (I) or negative (I) on the respective species [columns (i-iv)]. a) The species with high value has higher or similar abundance to the species with low value. If the impacts I and I have similar orders of magnitudes or |I| > |I|, scenario (a,ii) generates positive consequences (C₊) because $V_{high} \times N_{high} > V_{low} \times N_{low}$. Similarly, if the impacts I₊ and I have similar orders of magnitudes or |I| < |I|, scenario (a,iii) generates negative consequences (C-). If $|I_{\perp}| |I_{\perp}|$ or $|I_{\perp}| > |I_{\perp}|$ (for scenarios (a,ii) and (a,iii) respectively), the difference of impact can counter-balance $V_{\text{high}} \times N_{\text{high}} > V_{\text{low}} \times N_{\text{low}}$, making desirable consequences undesirable and vice versa. However, the difference of magnitude between I and I at which this switch occurs is difficult to determine due to the different units of V, N, and I. This uncertainty corresponds to a moral dilemma due to a conflict between the desire to have a small positive impact on the species with the larger value and abundance, and the desire to avoid a very negative impact on the species with the lower value and abundance for scenario (a,ii). For scenario (a,iii), the dilemma is due to a conflict between the desire to avoid a small negative impact on the species with the higher value and abundance, and the desire to have a very positive impact on the species with the lower value and abundance. b) The species with higher value V_{hieh} has the lower abundance N_{low}. If impacts are different between the two species, the opposition between V and N will most likely generate moral dilemmas (C,). If $V_{hieh} \times N_{low} > V_{low} \times N_{hieh}$, scenario (b,ii) is equivalent to scenario (a,ii), and to scenario (a,iii) otherwise (and scenario (b,iii) is equivalent to scenario (b,iii), and to scenario (a,ii) otherwise), but because value and abundance have different units, it is difficult to determine for which value and abundance $V_{hieh} \times N_{low} = V_{low}$ × N_{hieh}. Therefore, an additional moral dilemma arises due to a conflict between the desire to avoid a negative impact on the larger population and the desire to avoid a negative impact on the species with the higher value.

not to prescribe how such a constructed attribute should be computed, nor to be used directly as a decision analysis tool (i.e. not to be applied directly). To be used in such a fashion, constructed attributes need to be unambiguous, comprehensive, direct, operational, and understandable by the general public (Keeney and Gregory 2005). Because

value systems can be complex, meeting all five criteria is necessarily difficult. Instead, Equation 1 should be seen as a guide to ask questions that are relevant if management shall account for different value systems. By trying to evaluate Equation 1, one will have to ask such questions in a systematic fashion (Table 2), while understanding how these questions are conceptually linked with each other.

If Equation 1 could be evaluated, for each measure of impact and each system of values, Equation 1 would produce relative rather than absolute values. The values of consequences C of a management action under different value systems and measure of impact cannot be directly compared with each other, because the unit and range of values of C can vary between value systems. Instead, Equation 1 can be used to rank a set of management actions for each value system or measure of impact based on their assessed consequences, to identify management actions representing consensus, compromises or conflicts amongst value systems.

Equation 1 is particularly useful to identify potential moral dilemmas, i.e. situations in which management options are conflicting under the same value system (Table 1). For example, if different types of impacts are considered simultaneously under a value system (e.g. economic vs cultural impacts, or lethal impacts vs. those causing suffering, see sections below), Equation 1 might rank management actions differently for these different impacts under the same system of moral values.

In some situations the implication of Equation 1 is clear. For example, if an impact is positive for a highly valued, highly abundant species, but slightly negative for a few individuals of another species that is not considered very important ($C = I_{+} \times V_{high} \times N_{high} + I_{-} \times V_{low} \times N_{low}$), the consequence will be positive (Fig. 3aii). However, if the magnitude of the negative impact is much higher than that of the positive impact ($|I_{+}|$ |I-|), the consequence can become negative. Similarly, if impact is negative for the species with the highest value and abundance, and positive for the other species

Element of	Question	Mathematical	Examples of interpretation
Equation 1		formulation	
V_{i}	What relative value do you	What is the	If a few species have a disproportionately high value compared to
	place on individuals of different	distribution of V_i ?	others, i.e. speciesism, the distribution of V_j is highly skewed. If
	species?		all species have a similar value, the distribution of V_j is even.
I_{i}	What measure(s) of impact do	What is the unit	If only individual survival matters, I _j can be quantified as the
	you consider?	of I? How to	probability of death, and assessed through surveys. If animal
		quantify I?	wellbeing matters, approaches based on physical aspect, stress,
			etc. can be used to quantify I_i .
a	Do you value individuals or	What is the value	If every individual is valued the same (regardless of which species
	species? If you value species,	of a? Is a positive	they are) then a=1. This means that common species will be
	should rare species have more	or negative?	more highly valued overall in the assessment of the conservation
	values than common ones?		action. If all species are valued the same (regardless of differences
			in abundance) then a=0. This means that individuals of a rare
			species will be more highly valued than individuals of a common
			species in direct proportion to the abundance of the species. If
			rare species are valued more than common ones then a<0.

Table 2. Set of questions to ask in order to evaluate Equation 1 and related concepts. The purpose is to guide users in exploring all the elements to consider when assessing the consequences of management actions rather than necessarily attempting a quantification of each. See Table 3 for factors to consider to answer these questions.

 $(C = I_{\perp} \times V_{high} \times N_{high} + I_{\perp} \times V_{low} \times N_{low})$, the situation is clear if positive and negative impacts have the same magnitude, but it will shift once the magnitude of the positive impact becomes higher than the magnitude of the negative impact ($|I_i| > |I_i|$; the difference of magnitude will likely be lower than in the first example, because of the differences in sign; Fig. 3aiii). Since impact, value and abundance have different units, the thresholds at which these shifts occur are difficult to assess, and so the consequences can be highly debatable. This can create moral dilemmas, e.g. between the desire to have a small positive impact on a larger population with higher value and the desire to avoid a very negative impact on the species with the lower value and abundance (Fig. 3aii); and between the desire to avoid a small negative impact on the larger population with the higher value and the desire to have a very positive impact on the species with the lower value and abundance (Fig. 3aiii). Moral dilemmas will be even more likely to occur if the species with the higher value has the lower abundance $(C = I_{+} \times V_{high} \times N_{low})$ + $I \times V_{low} \times N_{high}$ or $C = I \times V_{high} \times N_{low} + I_{\star} \times V_{low} \times N_{high}$; Fig. 3bii,iii). If $V_{high} \times N_{low}$ > $V_{low} \times N_{high}$, the example depicted in Fig. 3bii is equivalent to the example depicted in Fig. 3aii described above, and Fig. 3biii is equivalent to the example depicted in Fig. 3aiii. If $V_{high} \times N_{low} < V_{low} \times N_{high}$, the example depicted in Fig. 3bii is equivalent to the example depicted in Fig. 3aiii described above, and Fig. 3biii is equivalent to the example depicted in Fig. 3aii. As above, it is difficult to determine when the inequality will change direction because of the difference in the units of V and N. This reflects a moral dilemma due to a conflict between the desire to avoid a negative impact on the larger population and the desire to avoid a negative impact on the species with the higher value. In summary, uncertainty in the computation of Equation 1, and in particular the need to compare parameters with different units (i.e. impact, value, and abundance), can therefore be interpreted as a moral dilemma (Fig. 3).

In addition, some actions might not follow moral norms compared to others despite having more desirable consequences. For example, killing individuals may be considered less moral, but more efficient to preserve biodiversity or ecosystem services than using landscape management. Solving these moral dilemmas is complex, and beyond the scope of this publication, but approaches such as multi-criteria decision analyses (MCDA; Huang et al. 2011) may offer an avenue to do so (Goetghebeur and Wagner 2017).

Similarly, environmental conflicts will likely emerge when comparing the rankings generated by Equation 1 under different value systems considering different distributions of values, and different measures of impact. MCDA (Wittmer et al. 2006) and operational research (Kunsch et al. 2009), have also been proposed to resolve such conflicts. We nonetheless argue that, regardless of the capacity to resolve environmental conflicts (or moral dilemmas), Equation 1 can help decision makers to understand how conflicts (might) emerge.

In the following, we discuss the complexity of assessing the different variables and parameters of Equation 1 under different value systems using the set of primary questions defined above. By doing so, it becomes possible to identify ambiguity, difficulty of operationality, etc., to eventually move towards a good constructed attribute (although such a constructed attribute may not be reached in practice). We also discuss how, despite the difficulty to quantify the variables described above, this framework can be used as a heuristic (rather than operational) tool to capture the implications of considering different value systems for determining the appropriateness of a conservation action, and to better understand conservation disputes.

Nature despite/for/and people

Over the past decade there has been some debate between proponents of traditional conservation, and those of new conservation (Table 1), as each group assumes different relationships between nature and people. Here, we show how the formal conceptualisation of Equation 1 could help clarifying the position of the new conservation approach in response to its criticisms (Kareiva 2014).

Nature despite people and traditional conservation

Traditional conservation is based on an ecocentric value system and seeks to maximise diversity of organisms, ecological complexity, and to enable evolution (Soulé 1985). For the sake of simplicity, we will consider an extreme perspective of traditional conservation, championed by 'fortress conservation' (Siurua 2006; Büscher 2016), i.e. excluding humans from the moral community. To capture these aspects, consequences *C* in Equation 1 can be more specifically expressed as follows:

$$C = \sum_{\text{species s (excluding humans)}} \bar{I}_s \times V_s \times N_s^{a < 0}$$
 Eq. 2

Assigning a stronger weight to rare species (a < 0) accounts for the fact that rare species are more likely to go extinct, decreasing the diversity of organisms. Evolution and ecological complexity are not explicitly accounted for in Equation 2. To do so, one may adapt Equation 2 and consider lineages or functional groups instead of species as the unit over which impacts are aggregated.

Because traditional conservation seeks to maximise diversity, I_s can be defined as the probability of individuals dying. $I_s \times N_s^{a < 0}$ will then be proportional to the extinction risk of a species (for an operational application, a proper model for extinction probability could be used in lieu of $I_s \times N_s^{a < 0}$). The V_s distribution could be considered uniform over all species, in the absence of biases.

Nature for people and new conservation

New conservation considers that many stakeholders ("resource users", Kareiva, 2014) tend to have an anthropocentric value system, and that conservation approaches that do not incorporate such a perspective will likely not succeed at maximizing diversity of organisms (Kareiva and Marvier 2012; Kareiva 2014). Under

anthropocentrism, species are only conserved due to their utilitarian value, i.e. their effect on I for humans, rather than based on an inherent value V. Different groups of stakeholders are likely to be impacted differently (e.g. different monetary benefits / losses), and we propose the following extension of Equation 1 to account for this variability:

$$C = \sum_{\text{stakeholders t}} \bar{I}_t \times V_t \times N_t$$
 Eq. 3

where \bar{I}_t is the average impact of management on the group of stakeholders *t*, including indirect impacts through the effect of management of non-human species. \bar{I}_t can correspond to economic impacts, or encompass categorical measures of wellbeing (e.g. Bacher et al. 2018). V_t is the value of the group of stakeholders *t*, and N_t is its abundance (i.e. the number of people that compose it). Parameter *a* is set to 1; as this is considered to be an individual-based value system. Note that including inherent values V_t in Equation 3 does not imply that we consider that different humans should be valued differently — though that is a view that some people hold — this needs to appear here to capture the full spectrum of perceived consequences of a management action.

New conservation holds an ambiguous perspective, stating that it should make "sure people benefit from conservation", while at the same time it does not "want to replace biological-diversity based conservation with a humanitarian movement" (Kareiva 2014). Using our framework, we interpret this to mean that one can design management actions that minimise consequences C under both Equations 2 and 3 (i.e. a mathematical expression of the convergence hypothesis; Norton 1986). Importantly, minimising Equation 3 is thereby a prerequisite for minimising impacts I and hence consequences C in Equation 2 (Fig. 2). Under this interpretation of new conservation, Equation 2 may therefore be rewritten as follows:

$$C = \sum_{\text{species s (excluding humans)}} \bar{I}_{s}(C_{humans}) \times V_{s} \times N_{s}^{a < 0}$$
 Eq. 4

where C_{humans} is computed using Equation 3, and assuming a monotonic and positive relationship between I_s and C_{humans} .

The link between biodiversity and ecosystem services is strongly supported, even if many unknowns remain (Chivian and Bernstein 2008; Cardinale et al. 2012), implying that high biodiversity can indeed support the provision of ecosystem services to humans. Such an approach will necessarily distinguish between "useful" species and others, and impacts will be perceived differently by different groups of stakeholders. Considering multiple types of impacts (economic benefits/losses, access to nature, health, etc.) while accounting for cultural differences, would increase the pool of useful species (comparing the resulting equation outputs using, for example, MCDA). The outcome of the two approaches would then potentially be more aligned with each other. This broad utilitarian perspective is captured in the most recent developments of new conservation approaches, which consider a wide range of nature contributions to people, rather than just ecosystem services (Díaz et al. 2018).

People and nature

People and nature views seek to simultaneously benefit human wellbeing and biodiversity (Fig. 2). Under this perspective, Equations 2 and 3 should therefore be computed separately (instead of being linked together as in Equation 4), before being combined in a single approach, for example using MCDA (Huang et al. 2011; assuming these equations can indeed be operationally computed), to capture a more diverse set of value systems than Equations 2 and 3 alone, even if the two approaches generate divergent results.

We expressed traditional and new conservation with Equations 2, 3 and 4, which correspond to extreme interpretations of these two approaches (excluding humans or considering specific utilities of species). Doing so illustrates how our mathematical framework can capture the pitfalls of failing to explicitly define normative postulates for conservation approaches. As a result, Equations 2, 3 and 4 will likely generate conflicting results in the ranking of different management actions, especially if few types of impacts are considered. The debates over new conservation have taken place in a discursive fashion, which has not provided a clear answer to the values defended by this approach (Kareiva 2014; Soulé 2014; Doak et al. 2015). It has therefore been argued that the normative postulates of new conservation need to be more clearly defined (Miller et al. 2011). Our framework could help doing so, by being explicit about how new conservation would be defined relative to the traditional conservation and the people and nature perspective through the addition of specific terms to Equation 3 and a thorough comparison of the resulting equations. In particular, it would be interesting to explore, how inherent values are attributed to different species under a new conservation approach compared to under a traditional conservation approach (e.g. relational vs. intrinsic value; Chan et al. 2016; Table 1) and how their distributions differ.

The case of animal welfare

The question of if and how animal welfare should be integrated into conservation practice is increasingly debated (Hampton and Hyndman 2018). Recently, conservation welfare (Table 1) has proposed to consider both the "fitness" (physical states) and "feelings" (mental experiences) of non-human individuals in conservation practice (Beausoleil et al. 2018). Based on virtue ethics rather than consequentialism, compassionate conservation (Table 1) also emphasises animal welfare and is based on the "growing recognition of the intrinsic value of conscious and sentient animals" (Wallach et al. 2018). It opposes the killing of sentient invasive alien species (Table 1); the killing of sentient native predators threatening endangered species; and the killing of sentient individuals from a given population to fund broader conservation goals.

Despite the near-universal support of conservation practitioners and scientists for compassion towards wildlife and ensuring animal welfare (Russell et al. 2016; Hayward et al. 2019; Oommen et al. 2019), compassionate conservation has sparked vigorous responses (Hampton et al. 2018; Driscoll and Watson 2019; Hayward et al. 2019; Oommen et al. 2019; Griffin et al. 2020). Amongst the main criticisms **Table 3.** List of factors to consider regarding the effects of environmental management actions from an environmental ethics perspective.

Factor	Influence on variables and outputs in Equations 1 to 5			
Biotic interactions	The impact or suffering of individuals from one species can be caused by individuals from another species,			
	either through direct or indirect interactions. Management actions can therefore also have non-trivial			
	indirect impacts on some species.			
Capacity to provide	The presence of a specific species may increase the fitness/welfare of other species through the ecosystem			
ecosystem services	services it provides. Since these effects can be difficult to quantify explicitly, the value of such species may be			
	increased in Equations 1 to 4 to account for them.			
Discounting rate	Rate at which impacts that occur in the future lose importance.			
Impact quantification	How the impacts of management actions are quantified is dependent on value systems, as some impacts			
and commensurability	(such as death) may be considered incommensurable to others (such as suffering).			
Responsibility from	Previous human actions on certain species, such as reintroduction of domesticated species or the			
previous actions	introduction of alien species , obviously can have had a direct impact on these species, but can also change			
	the perception of the public and therefore change the inherent value attributed to these species or change the			
	morality of an action.			
Spatial scale	The spatial scale will change the abundance N and the number of species considered. As a result, a			
	management action that is more beneficial than another at a small scale may not be such at a larger scale,			
	and vice versa. Additionally, the spatial scale can change the inherent value of species, for example under			
	nativism, or because of the range of cultures that are considered.			
Temporal scale	The time frame over which the impact or the suffering of individuals is computed can change their values.			
	Management actions may decrease welfare of individuals in the short term, but be beneficial in the long term			
	once the ecosystem has stabilised. Similarly, not culling some population may cause less suffering on the			
	short term, but increase it in the future by disrupting ecosystem services, leading to population collapse due			
	to lack of resources, etc.			
Uncertainty of impact	The complexity of an ecological system can make the impact of management actions on different species			
	difficult to assess precisely, therefore creating potential errors, especially in the presence of multiple biotic			
	interactions. This may lead to an incorrect estimation of the consequences C.			
Uncertainty of value	Quantifying the value given by a person or a group of people to an individual is difficult, context-dependent,			
expressions and	and highly subjective. Sensitivity analyses on the distribution of values can be used to account for such			
preferences	uncertainty.			

of compassionate conservation is that the absence of action can result in (often well understood and predictable) detrimental effects and increased suffering for individuals of other or the same species (including humans), as a result of altered biotic interactions across multiple trophic levels, i.e. "not doing anything" is an active choice that has consequences (Table 3). However, since compassionate conservation is not based on consequentialism, it uses different criteria to assess the appropriateness of conservations actions (but see (Wallach et al. 2020) for responses to some criticisms). Our purpose here is not to discuss the relevance or irrelevance of virtue ethics for conservation (see (Griffin et al. 2020) for such criticism). Instead, we propose discussing animal welfare from the perspective of consequentialism (Hampton et al. 2018), i.e. more aligned with the approach of conservation welfare (Beausoleil et al. 2018), and to show how it may be aligned with or oppose the traditional and new conservation approaches.

A mathematical conceptualisation of animal welfare

A consequentialist, sentientist perspective aims at maximizing happiness, or conversely minimising suffering, for all sentient beings, an approach also termed 'utilitarianism' (Singer 1980; Varner 2008). Suffering is therefore considered as a measure of impact

(or, in mathematical terms, impact is a function of suffering, which can be expressed as I(S) in Equation 1).

It has become widely accepted that animals experience emotions (de Waal 2011). Emotions have been shown to be linked to cognitive processes (Boissy and Lee 2014), which differ greatly among species (MacLean et al. 2012), and behavioural approaches have been used to evaluate and grade emotional responses (e.g. (Désiré et al. 2002); but see (Shriver 2006) and (Bermond et al. 2001) for different conclusions about the capacity of animals to experience suffering). We therefore postulate that the quantification of suffering is conceptually feasible in the context of the heuristic tool presented here. In a utilitarian approach, the inherent value of a species would therefore be a function of its capacity to experience emotions and suffering E_s , which can be expressed as V(E) instead of V_c in Equation 1.

Under these considerations for defining impact and value of species, the consequences of a conservation action can be computed as a function of suffering of individuals from species s, their capacity to experience emotion and suffering E_s , and the abundance of species s:

$$C = \sum_{\text{species s}} \overline{I(S_s)} \times V(E_s) \times N_s^{a=1}$$
 Eq. 5

Although V(E) should be measured in an objective fashion, many factors may influence the relationship between the inherent value and the emotional capacity of a species. For example, high empathy (Table 1) from the observer will tend to make the distribution uniform, whereas anthropomorphism and parochialism (Table 1) may lead to higher rating of the emotional capacities of species phylogenetically close to humans or with which humans are more often in contact, such as pets. Finally, we assumed that a = 1, to give equal importance to any individual regardless of the abundance of its species, as suffering and wellbeing are usually considered at the individual level (Beausoleil et al. 2018).

Assessing suffering in the presence and absence of conservation management actions

The short-term suffering resulting from pain and directly caused by lethal management actions, such as the use of poison to control invasive alien species (Twigg and Parker 2010) or the use of firearms and traps to cull native species threatening other native species (Proulx et al. 2016) or humans (Gibbs and Warren 2015), is the most straightforward type of suffering that can be assessed, and is usually sought to be minimised in all conservation approaches. Suffering can have many other causes, and suffering of an individual may be assessed through a wide variety of proxies, including access to food and water, death, number of dead kin for social animals, physiological measurements of stress hormones, etc. Suffering can take various forms, and commensurability can be an issue (Table 3), making the distinction between the morality of lethal actions and non-lethal

suffering complex. Non-lethal suffering can result from unfavourable environmental conditions (e.g. leading to food deprivation) and occur over long periods, while lethal actions could be carried out in a quick, non-painful fashion (Shao et al. 2018), and even lead to improved animal welfare (Wilson and Edwards 2019), but may be deemed immoral.

The concept of animal welfare and how to measure it is extremely complex (Beausoleil et al. 2018), and defining it precisely is beyond the scope of this study. We nonetheless advocate a conceptual approach that takes into account indirect consequences of management actions within a certain timeframe and consider uncertainty (Table 3). Direct and indirect biotic interactions may be explicitly modelled to quantify the impact on animals and their suffering. Simulation models can also make projections on how populations may change in time, accounting for future suffering.

Are traditional conservation and animal welfare compatible?

It has been argued that sentientism and ecocentrism are not fully incompatible (Varner 2011). The relationship between biodiversity and animal suffering can be formalised more clearly using the traditional conservation and the sentientist Equations 2 and 4, to explore if the same management action can minimise the consequences evaluated using the two equations (see also Suppl. material 1: Appendix S2 for the application of the framework to theoretical cases). The main difference with the traditional vs new conservation debate here is that Equations 2 and 4 share a number of species, whereas the new conservation Equation 3 only contains humans, which are excluded from Equation 2. Even though the variables of Equation 5 differ from those of Equation 2 (V and I are computed differently, and the value of a is different), it is possible that these equations will vary in similar ways for different management actions due to their similar structure, although this would depend on the variety of impacts on humans that are considered in Equation 3. Finally, as for the people and nature approach, the consequences of sentientist and ecocentric approaches can be evaluated in combination, as suggested by conservation welfare (Beausoleil et al. 2018), using tools such as MCDA (Wittmer et al. 2006; Huang et al. 2011).

One issue that may be irreconcilable between ecocentric approaches such as traditional conservation and approaches based on sentientism is the fate of rare and endangered species with limited or no sentience. Under utilitarian sentientism, the conservation of non-sentient species ranks lower than the conservation of sentient species, and consequently they are not included in Equation 5. For example, endangered plant species that are not a resource for the maintenance of sentient populations would receive no attention, as there would be few arguments for their conservation. Traditional conservation would focus on their conservation, as they would have a disproportionate impact in Equation 2, due to low abundance leading to a high value for $N^{a < 0}$.

Finally, it is important to note that the current body of knowledge shows that the link between biodiversity and animal welfare mentioned above especially applies to the increase of native biodiversity. Local increase of biodiversity due to the introduction of alien species may only be temporary due to extinction debt (Kuussaari et al. 2009) and often results in a reduction of ecosystem functioning (Cardinale et al. 2012). Therefore, it is important to distinguish between nativism (Table 1) and the detrimental effects of *invasive* alien species on biodiversity and ecosystem functioning and services (Bellard et al. 2016). Nativism would result in increasing the inherent value V_s of native species (Fig. 1), whereas in the second case, insights from science on the impact of invasive alien species would modify the distribution $I(S_s)$ rather than the distribution V_s . This can also apply to native species whose impacts on other species, such as predation, are increased through environmental changes (Carey et al. 2012).

Unresolved questions and limitations

From an operational perspective, this framework shares similarities with mathematical approaches used in conservation triage (Bottrill et al. 2008), but has two crucial differences. First, conservation triage is an ecocentric perspective with variables that are comparatively easy to quantify. Bottrill et al. (2008) provided an example using phylogenetic diversity as a measure of value *V*, and a binomial value *b* to quantify biodiversity benefit that can be interpreted as the presence or absence of a species (i.e. I = 1 / b). Because it is ecocentric, local species abundance is not considered, which corresponds to setting a = 0. In this example, consequences (*C*) in the general Equation 1 are therefore defined simply by V / b.

In contrast, our framework allows more flexibility to encompass a range of value systems, as shown above. However, given that the data needed for quantifying parameters of Equations 1 to 5 related to value, impact, emotional capacity and suffering are scarce and often very difficult to measure, this framework in its current form would be difficult to use as a quantitative decision tool to evaluate alternative management actions, contrary to triage equations. Rather, our equations decompose the question underlying many controversies around management decisions in conservation: what or who is valued, how, and to what extent?

Despite the difficulty to apply the framework, it can guide the search for approaches that may be used to develop quantification schemes for the different parameters of the framework and therefore obtain a better appreciation of the different facets of the valuation of nature. For example, grading systems may be developed to assess impact and suffering based on various indicators, including appearance, physiology, and behaviour (Broom 1988; Beausoleil et al. 2018). For assessing the value of different species, questionnaires may be used to assess how different species are valued by people, and influenced by their social and cultural background, similar to what has been done to assess species charisma (Colléony et al. 2017; Albert et al. 2018). It will nonetheless be important to acknowledge the corresponding uncertainties in the assessment of impact and value, differences in perception among societal groups for different taxa and potential shifts in perception over time (Table 3).

The second difference from conservation triage is that the latter considers additional criteria that were not addressed here, including feasibility, cost, and efficiency (including related uncertainties). The combination of these different perspectives calls for appropriate methods to include them all in decision making, which can be done using MCDA (Huang et al. 2011). Here, good communication and transparency of the decision process is key to achieve the highest possible acceptance across stakeholders, and to avoid biases in public perception (see case studies below for examples).

The issue of spatial and temporal scale also warrants consideration (Table 3). In the case of a species that may be detrimental to others in a given location but in decline globally, the spatial scale and the population considered for evaluating the terms of Equations 1 to 5 is crucial to determine appropriate management actions. Similarly, management actions may also result in a temporary decrease in welfare conditions for animals, which may increase later on (Ohl and Van der Staay 2012), or the impacts may be manifested with a temporal lag. In that case, determining the appropriate time period over which to evaluate the terms of Equations 1 to 4 will not be straightforward. Impacts will be of different importance depending on whether they occur in the short-or long-term, especially since long-term impacts are harder to predict and involve higher uncertainty. Discount rates (Table 3) may therefore be applied, in a similar way they are applied to the future effects of climate change and carbon emissions (Essl et al. 2018), or to assess the impact of alien species (Essl et al. 2017).

Equations 1 to 5 assume that all individuals from a given species have the same value or emotional capacities (or rather an average value is used across all individuals). However, intraspecific differences in value may be important for conservation. For example, reproductively active individuals contributing to population growth/recovery may be given a higher value in an ecocentric perspective. Trophy hunters might prefer to hunt adult male deer with large antlers. Intraspecific value may also vary spatially, for example between individuals in nature reserves or in highly disturbed ecosystems. In such cases, Equation 1 may need to be adapted to use custom groups of individuals with specific values within species, similar to Equation 3.

Finally, it is crucial to account for biotic interactions in our framework to comprehensively assess the indirect impacts of management actions on different species (Table 3). Some species with low values V_s in a certain value system may be crucial for assessing the impact I_s on other species. These biotic interactions will therefore determine the time frame over which the framework should be applied, as impacts on one species at a given time may have important repercussions in the future. These biotic interactions can be complex, and several tools, such as simulation models and ecological network analyses can be used to capture them. Concepts such as keystone species (Mills et al. 1993) can also offer a convenient way to overcome such complexity by modifying V_s rather than I_s . Let us assume that a management action will have a direct impact on a keystone species, which will result in indirect impacts on multiple other species with inherent values. Increasing the value of the keystone species can result in the same assessment of C as to explicitly model the biotic interactions and compute the resulting indirect impacts I_s .

Case studies illustrating ethical conflicts in conservation decisions

In the following, we present three case studies where conservation actions have either failed, had adverse effects, or were controversial, and we explore how our framework can help to identify normative postulates underlying these situations. Although these case studies have been discussed at length in the articles and reports we cite, we argue that our framework helps capture the different components of the controversies in a more straightforward and objective fashion than using a discussive approach that might require either emotionally loaded language or more neutral but less understood neologisms.

Invasive alien species management: the case of the alien grey squirrel in Italy

The grey squirrel (*Sciurus carolinensis*) is native to North America and was introduced in various locations in Europe during the late nineteenth and the twentieth century (Bertolino 2008). It threatens native European red squirrel (*Sciurus vulgaris*) populations through competitive exclusion and as a vector of transmission of squirrel poxvirus in Great Britain (Schuchert et al. 2014). Furthermore, it has wider impacts on woodlands and plantations, reducing the value of tree crops, and potentially affects bird populations through nest predation (Bertolino 2008).

Based on the impacts of the grey squirrel, an eradication campaign was implemented in 1997 in Italy, with encouraging preliminary results (Genovesi and Bertolino 2001). However, this eradication campaign was halted by public pressure from animal rights movements. The strategy of the animal rights activists consisted of (i) humanising the grey squirrel and using emotive messages (referring to grey squirrels as "Cip and Ciop", the Italian names of the Walt Disney "Chip and Dale" characters) and (ii) minimising or denying the impact of grey squirrels on native taxa, especially on the red squirrel (Genovesi and Bertolino 2001). In addition, the activists did not mention (iii) the difference in abundance between a small founding population of grey squirrels that could be eradicated by managers, and a large population of native red squirrels that would be extirpated or severely impacted by grey squirrels if control was not implemented.

Genovesi and Bertolino (2001) explain that the main reason for the failure of the species management was a different perspective on primary values. The conservation managers, favouring eradication, based their decision on species valuation, following traditional conservation. The animal rights activists, opposed to control, focussed on animal welfare. Applying the framework, and assuming an individual-based value system (a = 1 in Equation 1), three questions are apparent (Table 2):

- i. Are red and grey squirrels valued differently?
- ii. What types of impact are considered?

iii. Is the population of red squirrels impacted by grey squirrels larger than the population of grey squirrels to be controlled?

The arguments of animal rights activists led to the following answers to these three questions. (i) The humanisation of the grey squirrel consists of increasing the percep-

tion of its emotional capacity $E_{gs} > E_{rs}$ (and therefore $V(E_{gs}) > V(E_{rs})$). (ii) Minimising the impact of the grey squirrel is equal to restricting the time scale to a short one and to likely minimising the amount of suffering *S* caused by grey squirrels on other species (under a sentientist perspective), or the number of red squirrels that will die because of grey squirrels (under a biocentric perspective). In other words, $S_{gs} = S_{rs}$ (and therefore $I(S_{gs}) = I(S_{rs})$) or $I_{gs} = I_{rs}$ without management and $S_{gs} > S_{rs}$ (and therefore $I(S_{gs}) > I(S_{rs})$) or $I_{rs} > I_{rs}$ under management. (iii) Not mentioning differences in species abundance implies that the impacted populations of red and grey squirrels would have the same size under any management. Following these three points, the consequences under management $C_m = I(S_g) \times V(E_g) + I(S_r) \times V(E_r)$ are higher than without management, due to the increase in $V(E_g)$ and $I(S_g)$. The application of our framework therefore clarifies a discourse whose perception could otherwise be altered because of techniques such as an appeal to emotion.

The framework can thus be used to provide recommendations for what the advocates for the eradication campaign would have needed to have done: i) increase the value E_{r_s} of red squirrels in a similar way as what was done for grey squirrels, so that their relative values compared to grey squirrels would remain the same as before the communication campaign by the animal rights activists; ii) better explain the differences in animal death and suffering caused by the long-term presence of the grey squirrel compared to the short-term, carefully designed euthanasia protocol, which would avoid a subjective perception of the distribution of S; and iii) highlight the differences in the number of individuals affected. The consequences would then be computed as $C = V(E_g) \times I(S_g) \times N_g + V(E_g) \times I(S_g) \times N_g$. In that case, assuming the amount grey squirrels suffer as a result of being euthanised is the same as red squirrels suffer from the grey squirrels, and all squirrels (be they grey or red) are valued the same (i.e. avoiding nativism), the mere differences $N_{r_s} > N_g$ in abundance would lead to a higher value of C without management. This would further increase by extending the impacts of grey squirrels to other species, as mentioned above.

A more fundamental issue, however, is that in some value systems it would not be acceptable to actively kill individuals, even if that meant letting grey squirrels eliminate red squirrels over long periods of time (Wallach et al. 2018). The reluctance to support indirectly positive conservation programs is a common issue (Courchamp et al. 2017). Whether an acceptable threshold on consequences over which killing individuals could be determined through discussion would depend, in part, on the willingness of the affected parties to compromise.

De-domestication: the case of Oostvaardersplassen nature reserve

De-domestication, the intentional reintroduction of domesticated species to the wild, is a recent practice in conservation that raises new ethical questions related to the unique status of these species (Gamborg et al. 2010). Oostvaardersplassen is a Dutch nature reserve. Reserve managers, recognising that grazing by large herbivore was a key natural ecosystem process that had been lost, decided between 1983 and 1992 to reintroduce red deer (*Cervus elaphus*), and two domesticated species (Heck cattle,

Bos primigenius, and konik horses, *Equus ferus caballus*) (ICMO2 2010). The populations of these three species increased rapidly, as natural predators were missing and, as a result of a 'non-intervention-strategy', no active population control measures were implemented. The project was widely criticised when a considerable number of individuals died from starvation during a harsh winter, resulting in the subsequent introduction of culls.

From a traditional conservation perspective, disregarding animal welfare and focusing on species diversity and ecological restoration, the project was a success. The introduction of the three herbivore species led to sustainable populations (despite high winter mortality events), and ensured stability of bird populations without the need for further interventions (ICMO2 2010), i.e. the conditions of many species were improved (the impact was lowered), leading to improved consequences C for biodiversity overall (Equation 2). In other words, since more individuals from all species survived (I increased in Equation 2), C improved overall, regardless of differences in value or abundance between species (a multi-species generalisation of Fig. 3i).

However, the welfare of individuals from the three charismatic large herbivorous species became a point of conflict. In terms of the framework, it appears that the conflict was driven by considering the outcome of Equation 5 in addition to that of Equation 2 to estimate the overall evaluation of the management approach, i.e. a change from only considering impacts on individual survival to also considering impacts based on suffering, with the acknowledgement that E_s should be considered (Ohl and Van der Staay 2012). Not considering Equation 5 would mean that C = 0 under sentientism, but acknowledging the existence of E_s implies that $C = V(E_s) \times I(S_s) \times N_s^1$ becomes non-null. Changes in perspective over time should therefore be taken into account when implementing conservation management actions, and adaptive management approaches should be considered. A possible explanation for this shift in attitude is the notion of responsibility (Table 3). Culling animals might be acceptable in some cases, but might not be if these individuals were purposefully introduced, which may lead to considering a sentientist perspective.

The reserve managers examined a number of sustainable measures to improve the welfare of individuals from the three species (therefore decreasing S_j to compensate the increase in V_j). These included recommendations to increase access to natural shelter in neighbouring areas of woodland or forestry, to create shelter ridges to increase survival in winter as an ethical and sustainable solution, and to use early culling to regulate populations and avoid suffering from starvation in winter (ICMO2 2010). This example shows how a combination of two complementary management actions (the rewilding of the OVP and the provision of shelter) led to minimised consequences under both the traditional conservation and the sentientist Equations 2 and 5, whereas only rewilding would increase consequences under Equation 5. Interestingly, other approaches, such as the reintroduction of large predators, were also considered but discarded due to a lack of experience and too many uncertainties in efficiency (ICMO2 2010). Our suggested framework could be adapted to explore the consequences of culling vs. increased mortality through the reintroduction of

large predators, noting that some stakeholders may make moral distinctions between natural mortality and human-induced mortality. Culling may also still face opposition based on moral arguments.

Trophy hunting

Trophy hunting, the use of charismatic species for hunting activities, has been argued to be good for conservation when revenues are reinvested properly into nature protection and redistributed across local communities, but faces criticisms for moral reasons (Lindsey et al. 2007b; Di Minin et al. 2016). The action of killing some individuals to save others might be incompatible with a deontological perspective, but, assuming a consequentialist perspective, the framework can be applied to formalise the assessment of different management options. Note that here, we are not considering the ethics of how the hunt itself is carried out (e.g. canned hunting vs. a "fair chase") nor how animals are reared (i.e. whether they can express their natural behaviours), recognising that both these factors would need to be considered when making a decision.

In traditional conservation, trophy hunting is desirable if it directly contributes to the maintenance of species diversity. That is, it should decrease impacts I evaluated as individual survival over all or the majority of species with high inherent value, leading to improved consequences for biodiversity C in Equation 2 (a multi-species generalisation of Fig. 3i, ii). The potential of trophy hunting to contribute to the maintenance of biodiversity is via creating economic revenues, i.e. an anthropocentric perspective, and it therefore falls under the umbrella of new conservation (Fig. 2; Equation 4). In theory, trophy hunting should lead to lower consequences than doing nothing for both the traditional and new conservation (Equations 2, 3 and 4), and therefore for the 'people and nature' approach, as they are in this case not independent from each other (Lindsey et al. 2007a). Many social and biological factors currently affect the efficacy of trophy hunting as a conservation tool. Corruption and privatisation of the benefits have sometimes prevented the revenues to be reinvested into conservation, but also to be redistributed across local communities, whereas doing so has been shown to increase their participation in conservation actions with proven benefits for local biodiversity (Di Minin et al. 2016). In other words, a decrease in the anthropocentric Equation 2 leads to a decrease in the ecocentric Equation 3, but the causal link (Equation 4) is still supposed to be valid. In addition, trophy hunting can lead to unexpected evolutionary consequences (Coltman et al. 2003), overharvesting of young males (Lindsey et al. 2007b), and disproportionate pressure on threatened species (Palazy et al. 2011, 2012, 2013) and therefore to population declines and potential detrimental effects on biodiversity. That means that $I(C_{humans})$ in Equation 4 should be carefully examined. Despite these issues, it has been argued that banning trophy hunting may create replacement activities that would be more detrimental to biodiversity (Di Minin et al. 2016).

From an animal welfare perspective, trophy hunting appears to be in direct contradiction with a decrease in animal suffering, and has been criticised by proponents of compassionate conservation (Wallach et al. 2018). However, as for the culling of invasive alien species, we suspect the story is more complex. First, there may be direct benefits for animal welfare on average, if money from trophy hunting is reinvested in protection measures against poaching (if such poaching causes, on balance, more suffering). Second, to our knowledge, only a few studies have compared the welfare of individual animals to quantify the elements of the sentientist Equation 5 (for example, assessed through access to resources) in areas where trophy hunting is practised and where it is not. Given the links between biodiversity and animal welfare described above, it seems plausible that good practice in trophy hunting may benefit the welfare of individuals from other, and from the same, species.

Conclusions

A variety of value systems exist in conservation. These are based on different underlying normative postulates and can differ between stakeholders, resulting in differing preferences for conservation practices among people. Here, we have proposed a framework with a formal set of equations to conceptualise and decompose these different perspectives from a consequentialist point of view. In this framework, the different value systems supported by different conservation approaches follow the same structure, but can differ in the variables used, and in the values they take. Such a formalisation, by necessity, does not capture the full range of complex and nuanced real-world situations in environmental decision-making, and the elements of the equations can be difficult to estimate. However, this framework is not intended to be an operational approach readily applicable across all value systems. Rather, the mathematical structure and the systematic examination of the elements of the framework provides a method to make the underlying value systems and the resulting conflicts explicit and transparent, which is essential for the planning and implementation of pro-active management. The search for consensus in conservation can be counter-productive and favour statusquo or 'do nothing' against pro-active management (Peterson et al. 2005), however our framework may help identify hidden commonalities between seemingly antagonistic stances. We hope that this framework can foster fruitful debates and thus facilitate the resolution of contested conservation issues, and will ultimately contribute to a broader appreciation of different viewpoints. In an increasingly complex world shaped by human activities, this is becoming ever more important.

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Supplementary material I

Appendix S1, S2

Authors: Guillaume Latombe, Bernd Lenzner, Anna Schertler, Stefan Dullinger, Michael Glaser, Ivan Jarić, Aníbal Pauchard, John R. U. Wilson, Franz Essl

Data type: Docx. file

- Explanation note: Appendix S1. Formalisation of ecosystem-based ecocentrism. Appendix S2. Examples of conflicting situations between traditional and compassionate conservation.
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Demography of an invading forest insect reunited with hosts and parasitoids from its native range

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Abstract

The Sirex woodwasp *Sirex noctilio* Fabricius (Hymenoptera: Siricidae), a widespread invasive pest of pines in the Southern Hemisphere, was first detected in North America in 2004. This study assessed the impacts of life history traits, host resistance and species interactions on the demography of *S. noctilio* in New York, Pennsylvania and Vermont, then compared key metrics to those found in the native range in Galicia, Spain. Many trees naturally attacked by *S. noctilio* in North America produced no adult woodwasps, with 5 of 38 infested trees (13%) sampled across six sites yielding 64% of emerging insects. Reproductive success was highest in the introduced host scots pine, *Pinus sylvestris*, but native red pine, *Pinus resinosa*, produced larger insects. *Sirex noctilio* required one or sometimes two years to develop and sex ratios were male biased, 1:2.98 $\mathcal{P}:\mathcal{O}$. Body size and fecundity were highly variable, but generally lower than observed in non-native populations in the Southern Hemisphere. Hymenopteran parasitoids killed approximately 20% of *S. noctilio* larvae and 63% of emerging adults were colonized by the parasitic nematode *Deladenus siricidicola*, although no nematodes entered eggs. Demographic models suggested that *S. noctilio* in the northeastern USA have a higher potential for population growth than populations in the native range: estimated finite factor of increase, λ , was 4.17–4.52 (depending on tree species colonized), compared to $\lambda = 1.57$ in Spain.

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Keywords

Forest pest, invasive species, population ecology, Sirex noctilio, woodwasp

Introduction

Non-native insects are among the greatest current threats to global forest resources (Liebhold et al. 2017). Economic and ecological impacts in coming years are expected to be severe (Mack et al. 2000) as introduction rates increase with the intensification of human transport (Banks et al. 2015; Brockerhoff and Liebhold 2017). Despite efforts to develop tools that can identify important invasive species before they become established and spread (Roy et al. 2015; Matthews et al. 2017), anticipating the effects of invasions in new regions remains challenging (Simberloff et al. 2013). The difficulty of predicting future invasions is exacerbated by the fact that many organisms, whose presence goes largely unnoticed in some places, particularly within their native range, can become damaging pests in others.

One approach for identifying key drivers of impacts caused by alien species is to compare important demographic parameters across native and invaded ranges (Guo 2006; Broennimann and Guisan 2008; Ayres et al. 2014). Across disparate regions of a species' introduced range, population behavior may differ as a consequence of variation in key life history traits, population genetics or local adaptation, host identity, density and relative suitability, and/or the composition or abundance of key community members including mutualists, competitors and natural enemies, among other factors (Garnas et al. 2016). Identifying the demographic forces responsible for sometimes dramatic differences in pest abundance and aggressiveness is a difficult proposition.

The Sirex woodwasp, *Sirex noctilio* Fabricius (Hymenoptera: Siricidae), provides an ideal system for the comparison of invasive behavior across a range of contexts, with native populations in Eurasia and widely-studied non-native populations in both the Northern and Southern Hemispheres (Slippers et al. 2015). *Sirex noctilio* is one of the most prominent agents of tree mortality in Southern Hemisphere pine plantations in Australasia (Carnegie and Bashford 2012), South Africa (Hurley et al. 2007) and South America (Lantschner and Corley 2015). Like many introduced forest pests and pathogens, this species has minimal impacts in its native range (Spradbery and Kirk 1981; Ayres et al. 2014). Superficially at least, North American *S. noctilio* population dynamics appear to more closely resemble native Eurasian populations than highly damaging Southern Hemisphere populations (Gilbert and Miller 1952; Haugen 1990; Hurley et al. 2007).

Sirex noctilio was first detected in North America in a survey trap in Fulton, NY near Lake Ontario in 2004 (Hoebeke et al. 2005) and surveys during the following two years confirmed the presence of reproducing populations in 17 Canadian and 27 US counties (Dodds et al. 2007; Dodds and de Groot 2012). The wasp has since spread northwards into Ontario and Quebec, Canada and southwards into at least eight US states. *Sirex noctilio* mainly colonizes and reproduces in a wide range of hard pine (subgenus *Pinus*) hosts, though other pines and conifers are occasionally utilized

(Hoebeke et al. 2005). The primary susceptible pine hosts in the current US range of *S. noctilio* are the native red pine *Pinus resinosa* Aiton and the introduced European Scots pine *P. sylvestris* L. Both occur in small, isolated stands in the northeastern USA, largely as a legacy of plantings carried out by the Civilian Conservation Corps (CCC) in the wake of the Great Depression (Maher 2008). Many such stands are even-aged and have been minimally managed following establishment. They are frequently overstocked, stressed and in poor condition (Dodds et al. 2010; Zylstra et al. 2010). These stands often contained dying trees even prior to *S. noctilio* invasion and have little or no timber value (Ayres et al. 2014). Although the native Eastern white pine *P. strobus* is abundant in this region and has been shown to be a suitable host for *S. noctilio* larvae, female woodwasps are rarely attracted to it and the species has not been meaningfully impacted (Haavik et al. 2017). For these reasons, economic losses associated with *S. noctilio* spread in eastern North America have been minimal (Ayres et al. 2014; Haavik et al. 2016; Haavik et al. 2018).

Female *S. noctilio* oviposit in suppressed or weakened pine trees (Dodds et al. 2010; Ayres et al. 2014). At each oviposition site, females drill one or more tunnels that branch from a single entrance through the bark (Madden 1974; Spradbery 1977). These wounds typically result in resin exudation and the drippings that form on the outside of the tree are used as an indicator of *S. noctilio* attacks during surveys (Ayres et al. 2009). Into each oviposition tunnel, gravid females then deposit one or more of the following: eggs, a phytotoxic venom (Madden 1968) and oidia (spores) of the obligate mutualist fungus *Amylostereum areolatum* (Fr.) Boiden (Russulales: Stereaceae) (Francke-Grosman 1939; Madden and Coutts 1979). If tree defenses are overcome, *A. areolatum* establishment and rapid tree decline normally follow (Coutts 1969; Neumann et al. 1987; Slippers et al. 2012). Larvae develop and pupate inside the xylem (Ryan and Hurley 2012), with the majority emerging after one year in most populations (but see Morgan 1968).

A similar natural enemy complex is found in both the native range of *S. noctilio* and in North America. In North America, several native parasitoid species utilize both *S. noctilio* and native siricid species, such as *S. nigricornis* (Coyle and Gandhi 2012). These include *Ibalia leucospoides ensiger* (Norton) (Hymenoptera: Ibaliidae), *Rhyssa persuasoria* (L.) and *R. lineolata* (Kirby) (Hurley et al. 2007; Slippers et al. 2015). The rhyssines can, in turn, be parasitized by the kleptoparasitoid *Pseudorhyssa* spp. (Couturier 1949; Spradbery 1969). The parasitic nematode *Deladenus* (= *Beddingia*) *siricidicola* (Tylenchida: Neotylenchidae) has a bicyclic life cycle, with a free-living asexual stage that feeds on *Amylostereum spp.* fungi within a tree and a parasitic sexual stage that occurs in the presence of developing siricids. The parasitic form is characterized by altered morphology and enters larvae and ultimately the testes or ovaries of the adult wasps, entering and sterilizing the eggs of females which vector them to new trees (Bedding 1967, 1972). *Deladenus siricidicola* strains in North America appear to be non-sterilizing, however, with nematodes reaching the gonadal tissues, but not entering the eggs (Kroll et al. 2013).

One possible explanation for differences in the behavior and impacts of invasive species populations is variability in important life history traits that impact potential population growth via fitness and fecundity. Perhaps the most important of these are insect body size and sex ratio (Kajita and Evans 2010; Liu et al. 2017; Tabak et al. 2018). *Sirex noctilio* exhibits significant sexual dimorphism as well as an unusual degree of within-sex body size variation, with more than a 3-fold variation in female size reported in both Tasmania (Madden 1974) and Argentina (Corley et al. 2007). Sex ratio differences can also have profound effects on population demography and can influence outcomes of invasions (Xu et al. 2016), as well as their ecological consequences (Fryxell et al. 2015). *Sirex noctilio* sex ratios vary widely (Caetano and Hajek 2017), from relatively even (~ 1:3 $\mathfrak{Q}:\mathfrak{T}$) in the native range in Spain (Lombardero et al. 2016) to extreme male-biased populations (~ 1:32 $\mathfrak{Q}:\mathfrak{T}$) in Brazil (Iede et al. 1998). Since they are haplodiploid, unmated females produce exclusively male offspring via arrhenotoky (Gardner 2014). When males are scarce, such as in recently introduced or highly dispersed populations, sex ratios might be highly male-biased due to selective investment in sons (Queffelec et al. 2019) or lower mate-finding success among females.

Another potential explanation for variation in S. noctilio impacts is tree resistance or variation in the suitability of host trees, which has been hypothesized as a key factor controlling populations in North America (Haavik et al. 2017). This could differ as a function of species and provenance, planting density and management in plantation environments or stand density and history in the case of natural or semi-natural stands. This variation could be further mediated by environmental effects on plant investment in growth versus defense (Herms and Mattson 1992) or by genetic differences in susceptibility, attractiveness or suitability as a larval resource. If these factors lead to differences in host susceptibility between environments, both S. noctilio population behavior and tree mortality patterns would be impacted (Haavik et al. 2016a; Martinson et al. 2018). Sirex noctilio is known to mainly attack suppressed or weakened trees in its native range or when at sub-outbreak levels in invaded areas (Madden 1968). Early studies of S. noctilio outbreaks in New Zealand suggested that intermittent drought increased synchrony in the susceptibility of plantation trees and recent findings in Argentina support this hypothesis while further suggesting that drought-related impacts can vary dramatically among host tree species in a region (Lantschner and Corley 2015; Lantschner et al. 2019).

A third potential explanation for frequent *S. noctilio* outbreaks in certain environments is the lack of controls by natural enemies in some non-native populations (Keane and Crawley 2002; Boissin et al. 2012). Since the appearance of *S. noctilio* as a pest in the early 1900s in New Zealand, management efforts throughout its invaded range have been dominated by the development and adaptation of nematode-based biocontrol strategies (Hurley et al. 2007; Slippers et al. 2015). In Southern Hemisphere countries, both intentional (e.g., New Zealand, Australia, South Africa) and accidental (e.g., Argentina, Uruguay) release of hymenopteran parasitoids may help limit woodwasp populations (Fischbein and Corley 2014). Differences in parasitoids could contribute to differences in outbreak behavior among regions.

Our study was driven by the motivation to better understand current and potential future *S. noctilio* population dynamics in the eastern USA. Comprehensive research

efforts to date in the USA and Canada have generated a range of estimates for important demographic parameters in these expanding populations and we have summarized these estimates in Table 1. It nonetheless remains unclear which factors will most strongly influence the trajectory of *S. noctilio* populations. Whether the relatively low population densities, growth rates and impacts in North America will persist — particularly as ranges expand and new hosts are encountered — is an open question. In this study, we evaluated the relative influence of life history traits, interactions with natural enemies and host susceptibility on estimated *S. noctilio* population growth rates in the northeastern United States. We then compared these results to demographic analysis from a recent study in the native range in Galicia, Spain (Lombardero et al. 2016) in order to understand how woodwasp populations might be expected to behave in North American pine forests as *S. noctilio* range expansion continues.

Variable	Value	Location	Source			
Emergence	early July to early September	Ontario	Ryan et al. (2012)			
timing	July to September	Ontario	Haavik et al. (2013)			
	early July to early September	NY & PA	Foelker et al. (2016)			
	June to October	NY & PA	Hajek et al. (2017)			
	July to October	NY, PA, VT	This study			
Development	4.1% required more than 1 year	Ontario	Ryan et al. (2012)			
time	0.8% larvae remaining after year 1	central NY	Myers et al. (2014)			
	4% larvae remaining after year 1	NY & PA	Foelker et al. (2016)			
	1.5–26.7% required > 1 year	NY & PA	Hajek et al. (2017)			
	10.4% required 2 years	NY, PA, VT	This study			
Sex ratio	20.6% ♀	central NY	Long et al. (2009)			
	- 25% ♀	Ontario	Ryan et al. (2012)			
	27% ♀	central NY	Myers et al. (2014)			
	variable; 13.7% - > 60% ♀	Ontario	Haavik et al. (2016b)			
	25% ♀	NY, PA, VT	This study			
Body Size	pronotum width 3.2 mm (parasitized), 3.5 mm (non- parasitized)	NY & PA	Kroll et al. (2013)			
	pronotum width $\stackrel{\frown}{=} 3.2 \text{ mm}$, $\stackrel{\frown}{\circ} 2.2 \text{ mm}$	Ontario	Haavik et al. (2016a)			
	♀ prothorax 3.9 mm (non-parasitized)	NY & PA	NY & PA Hajek et al. (2017) NY. PA. VT This study			
	\bigcirc body 19.7 mm; prothorax 2.6 mm; head capsule 2.8 mm	NY, PA, VT	This study			
Fecundity	Average No. eggs: 79.6 (parasitized), 108.3 (non- parasitized)	NY&PA	Kroll et al. (2013)			
	No. eggs = 69·(pronotum width in mm)-96	Ontario	Haavik et al. (2016a)			
	No. eggs = $0.17 \cdot (body length in mm)^{2.072}$	NY, PA, VT	This study			
Ibalia	21%	central NY	Long et al. (2009)			
leucospoides	10.6% P. sylvestris, 10.8% P. resinosa	NY	Eager et al. (2011)			
parasitism	20.8%	Ontario	Ryan et al. (2012)			
	18%	central NY	Zylstra and Mastro (2012)			
	3.4–17%	Ontario	Haavik et al. (2015)			
	13%	NY & PA	Foelker et al. (2016)			
	0–46%	Ontario	Haavik et al. (2016b)			
	20%	NY, PA, VT	This study			

Table 1. Life history parameters reported in previous studies in North America. Summary of findings to date from all North American studies that have assessed aspects of *Sirex noctilio* life history. Variables include timing of emergence, voltinism, sex ratio, body size, fecundity, parasitism and larval survivorship.

Variable	Value	Location	Source
Rhyssine	1%	central NY	Long et al. (2009)
parasitism	4.4% P. resinosa, 8.3% P. sylvestris	NY	Eager et al. (2011)
	3%	Ontario	Ryan et al. (2012)
	10%	central NY	Zylstra and Mastro (2012)
	12%	NY & PA	Foelker et al. (2016)
	0-6%	Ontario	Haavik et al. (2016b)
	3.5%	NY, PA, VT	This study
Total	21.8%	Central NY	Long et al. (2009)
hymenopteran	16.4%	NY	Eager et al. (2011)
parasitism	23.4%	Ontario	Ryan et al. (2012)
	41.2%	NY & PA	Kroll et al. (2013)
	1–50% range	Ontario	Haavik et al. (2016b)
	23.4%	NY, PA, VT	This study
Deladenus	38% of \mathcal{Q} , no sterilization	Ontario	Ryan et al. (2012)
parasitism	0.2–3% in larvae	central NY	Zylstra and Mastro (2012)
	27.9% all individuals	NY & PA	Kroll et al. (2013)
	23.7% all individuals	NY & PA	Foelker et al. (2016)
	32–64% of \bigcirc , 23–62% of \eth	Ontario	Haavik et al. (2016a)
	23.6% of \mathcal{Q} ; higher in <i>P. sylvestris</i> than <i>P. resinosa</i>	NY	Williams and Hajek (2017)
	62%	NY, PA, VT	This study
Survivorship	~ 1–14% larval survivorship	Ontario	Haavik et al. (2015)
(egg to adult)	30% larval mortality within tree	NY	Foelker (2016)
	~ 5%	Ontario	Haavik et al. (2017)
	28%	NY, PA, VT	This study

Materials and methods

Study area and materials

We collected S. noctilio in New York, Pennsylvania and Vermont in 2013, 2014 and 2015. We searched for stands of hard pines (P. resinosa and P. sylvestris) during spring and summer, noting the locations of trees with emergence holes and resin drippings indicating S. noctilio attack in the previous season. Trees attacked by S. noctilio were difficult to find, with > 80% of stands showing no signs of woodwasp presence. In total, we located active S. noctilio populations in six locations in New York (MF: Montour Falls, Schuyler County, 42.3354°N, 76.8138°W), Pennsylvania (DE: Delmar, Tioga County, 41.7209°N, 77.3772°W; MI: Middlebury, Tioga County, 41.8416°N, 77.4072°W, BL: State Game Lands 276 in Blacklick, Indiana County, 40.4886°N, 79.1070°W; CL: Clarion County, 41.1777°N, 79.2269°W) and Vermont (UN: Underhill, Chittenden County, 44.4847°N, 72.9656°W) across three years. As is common in the region, most sites primarily contained one tree species: three stands contained *P. sylvestris* only (MF, DE, MI), two stands contained P. resinosa only (BL, CL) and one stand contained both (UN), though *P. resinosa* was more abundant (Table 2). We recognize that species and site are necessarily confounded in this study due to the rarity of naturally-attacked hard pine stands on the landscape. We still chose to include comparisons of tree species in our analysis because each species occurred at multiple sites dispersed throughout the study area.

Year	State	County	No.	Tree Species	No. Sirex	No. Sirex	No. Ibalia	No. rhyssines
			trees		noctilio	nigricornis	leucospoides	
2013	NY	MF	3	P. sylvestris	1	0	0	0
	PA	DE	8	P. sylvestris	170	0	74	4
	PA	MI	3	P. sylvestris	0	0	0	0
	VT	UN	4	P. resinosa	71	68	0	30
2014	PA	DE	8	P. sylvestris	499	0	96	12
	VT	UN	1	P. sylvestris	2	9	0	1
	VT	UN	2	P. resinosa	77	66	2	16
2015	PA	BL	4	P. resinosa	41	0	0	0
	PA	CL	5	P. resinosa	130	0	67	3
Total			38		991	143	239	66

Table 2. Summary of trees and emerging insects sampled in each year of study. Number of trees sampled and number of native *Sirex nigricornis*, non-native *S. noctilio* and *Sirex spp.* parasitoids (*Ibalia leucospoides* and the rhyssines) collected from each tree species, at each site, across sampling years.

During late June or early July 2014–2016, we visited previously-identified sites to cut trees attacked in the prior season. All trees were dead or dying at the time of cutting. Over three years, we felled 38 attacked trees, recording GPS locations and diameter at breast height (dbh) for each. After felling, we cut each stem into ~ 1 m-long bolts, discarding the top of the tree (diameter < ~ 2 cm). Bolts were labeled individually to record their position relative to the ground and transported to emergence containers at Dartmouth College, Hanover, NH.

Insect emergence

Wasp-infested logs were placed in 55-gallon laminated cardboard emergence drums and stored indoors (ambient laboratory temperature ~ 21 °C). Drum openings were covered with fine mesh to prevent insect escape. Drums were checked every 1-3 days during peak emergence (mid-July to September) and at least twice a week thereafter until several weeks had passed with no new emergences (late October or early November). At the end of the first emergence season, we removed dead insects, then stored bolts in the laboratory until the following May, at which point we resumed regular checks. All emerging insects were collected, including S. noctilio, the native S. nigricornis and hymenopteran parasitoids. Due to low overall numbers, rhyssine parasitoids (R. persuasoria and R. lineolata) and kleptoparasitoids Pseudorhyssa spp. were combined for analysis and referred to as "rhyssines". For S. noctilio and S. nigricornis, we measured body length (excluding the ovipositor), then dissected each individual to check for nematodes. Nematodes from a subset of wasps were cultured and confirmed as *D. siricidicola* when they were sequenced as part of a study by Fitza et al. (2019). For 176 of the females, we counted the total number of eggs and, for 36 haphazardly selected individuals, we measured the length of five eggs per female using a compound microscope.

Bolt measurements and dissections

Collected bolts were weighed and measured for length and diameter at each end. For each bolt, we calculated the surface area and volume from length and diameter. Before we placed them in emergence drums, we measured wood moisture content at five locations along each bolt (~ 0.8 cm depth) using a Delmhorst RDM-3 moisture meter (Delmhorst Instrument Co., Towaco NJ) and averaged these measurements for analysis. Occasional measurements above or below the operating range of the device (6–60%) were recorded as 6% and 60%, respectively.

After insects finished emerging in the second year (at least two months with no further emergence), we dissected a subset of bolts (2-3 per tree). We counted resin drippings and emergence holes, then removed the bark and cambium to count oviposition sites (attacks) and the number of holes (drills) per attack using an illuminated tabletop magnifier $(5\times)$ and hand lens $(10\times-20\times)$ as needed, following methods established by Lombardero et al. (2016). For each attack, we noted the presence or absence of lesions (Suppl. material 1: Figs S1, S2), which are thought to be indicative of a polyphenol defense response by the tree (Coutts and Dolezal 1966; Lombardero et al. 2016). Since lesions were assessed two years after bolts were collected and may have faded over time, we considered this to be a conservative estimate of lesion formation.

For each bolt, we estimated the number of eggs laid, based on the number of attacks and drills per attack following Madden (1974) and Haavik et al. (2015):

eggs = 0.01 single drills + 0.68 double drills + 1.55 triple drills + 2.22 quadruple drills

After dissection, we cut each bolt into three equal lengths (avoiding knots) to expose fresh surfaces. We estimated the percentage of the cross-sectional area colonized by bluestain (ophiostomatoid) fungi by outlining visible bluestain on the cut surface of each bolt and photographing the surface (see Suppl. material 1: Fig. S2). Area quantification was performed using ImageJ as described in Lombardero et al. (2016).

Additional data

For analyses of *S. noctilio* body size, egg number and allometric equation development, we incorporated additional data from 1,511 emerging *S. noctilio*, collected from 53 trees sampled in central NY in 2008; hereafter referred to as the "Central NY data set" (see Zylstra et al. (2010) and Myers et al. (2014) for protocol details). These trees were chemically girdled in 2007 and felled after the oviposition season finished, at which point they were transported to the USDA APHIS PPQ laboratory (North Syracuse, NY) for storage in emergence barrels. Emerging insects were collected and the following measurements were recorded: hind tibial length, prothorax width, mass and egg count. As these trees were chemically girdled rather than naturally attacked, analyses were done separately from 2013–2015 data.

To compare egg sizes between the USA and Spain, we also measured eggs from *S. noctilio* collected emerging from bolts in Galicia, Spain in 2013–2015, as described in Lombardero et al. (2016). To further compare demographic patterns between the USA and Spain, we reanalyzed data used to build Table 1 in Lombardero et al. (2016), reproducing some parts of that Table and updating it with additional calculations of numbers of eggs laid, based on published estimates (Madden 1974) as described above.

Statistical analysis

Data analysis was conducted in JMP Pro 13.0 (SAS Institute 2016) and R Version 3.4.3 (R Core Team 2017). All means are reported with standard errors (SE) unless otherwise noted. In cases of unequal variance, we used Welch's Test to compare means. As we did not sample at all sites in all years, we included a composite variable Site-Year in some analyses.

We used Maximum Likelihood to estimate the number of wasps emerging per tree, number of insects emerging per bolt and the number of nematodes per parasitized *S. noctilio* adult. For each of these metrics, we evaluated four candidate distributions and compared them via log-likelihood: Poisson, zero-inflated Poisson, negative binomial and zero-inflated negative binomial (R package VGAM; Yee 2015).

We used Chi-square statistics to test for differences in: voltinism between male and female wasps; sex ratio between pine species and across sites; and nematode parasitism among tree species, sites and wasps of different sexes. We used restricted Maximum Likelihood generalized linear mixed models (R package lme4; Bates et al. 2015) to examine relationships between predictors of interest and emergence date, body size and egg length. We calculated *P* values using the Satterthwaite approximation for degreess of freedom (package lmerTest; Kuznetsova et al. 2017) and selected the best models by comparing AIC values (AICtab in bbmle package; Bolker et al. 2017) unless otherwise noted in results.

We used simple linear regression to test for a relationship between emergence date and body size, analyzing insects emerging from the *P. resinosa* and *P. sylvestris* separately. The impacts of sex and tree species on insect body size were examined using 2-way ANOVA with an interaction term. To describe the relationship between body size and *S. noctilio* egg number, we used the nls function in the R base package to fit a power function to our egg count data from dissected females, then tested for effects of tree species and voltinism on egg number by analyzing the residuals.

Parasitism rates were estimated as the slope of the regression line (with forced intercept = 0) of numbers of emerging parasitoids versus numbers of all emerging insects (siricids plus parasitoids) (Cochran 1977). We then used multiple regression to assess the effect of tree species on parasitism rate.

To examine relationships between bolt traits and measures of insect attack and emergence, we generated a matrix of Pearson Correlation Coefficients using all complete pairwise observations, then visualized them with the R package corrplot (Wei and Simko 2017) using an $\alpha = 0.05$ significance level. We transformed variables as noted

to address departures from normality and heterogeneity of variance. The logit transformations used a standard adjustment (Warton and Hui 2011) of 0.025 (R package CAR; Fox and Weisberg 2011). Patterns in drills per attack were assessed using a generalized linear mixed model with a Poisson link function, that included random effects of tree (nested within site) and bolt (nested within tree). We estimated emergence per attack and emergence per egg as the slope of a regression (with forced intercept = 0) of these pairs of variables (Cochran 1977).

Results

Sirex life history in NY,VT and PA

We collected 1007 *S. noctilio* (253 females and 754 males) over three years from 38 trees harvested in New York, Pennsylvania and Vermont (Table 2). The majority of *S. noctilio* emergence was concentrated in a small number of trees, with production per tree best described by a zero-inflated negative binomial (ZINB) distribution with $\Phi = 0.32$ (proportion of excess zeroes), $\mu = 75.39$ and overdispersion parameter k = 0.35 (Suppl. material 1: Fig. S3). Most wasps (89.6%) emerged within one year of bolt collection; the rest (10.4%) emerged in year two. Females were more likely than males to emerge in year 2 (21.7% vs. 6.6%; $\chi^2 = 40.53$, df = 1, p < 0.0001). Woodwasps emerged from 3 July to 2 November (Julian Days 184–306). This timing did not vary across years, but peak emergence date was slightly earlier for males than for females ($F_{1,839} = 3.74$, p = 0.053). Approximately 77% of the variation in emergence dates was among trees within a site.

The sex ratio of emerging *S. noctilio* was male biased 1:2.98 \bigcirc : \bigcirc . Voltinism influenced sex ratios, with insects emerging in one year having a sex ratio of 1:3.5 \bigcirc : \bigcirc and females dominating in year 2, with 1:0.90 \bigcirc : \bigcirc . Sex ratio also varied across sites, with the lowest proportion of males in a *P. resinosa* stand (Blacklick, Pennsylvania) and the highest at a *P. sylvestris* stand (Delmar, Pennsylvania; 0.32 vs. 0.83; $\chi^2 = 59.43$, df = 4, p < 0.0001). Although both of these sites are in Pennsylvania, the time since the stands had first become infested appeared to differ: *S. noctilio* had likely recently arrived to Blacklick, while a population was established in the area around Delmar since at least 2008 (Williams and Hajek 2017).

Sirex noctilio body length ranged from 6–37 mm, with an average length of 15.66 \pm 0.16 mm. Females (n = 251, 19.67 \pm 0.31 mm) were larger than males (n = 752, 14.32 \pm 0.15 mm) (t = 15.40, df = 369.63, p < 0.0001; Fig. 1) and insects emerging in the second year were larger than those emerging in the first ($F_{1,860.69} = 12.41$, p < 0.001). Year of emergence effects were consistent across sexes but more pronounced for males (1st year: 14.08 \pm 0.15 mm vs. 2nd year: 17.83 \pm 0.62 mm; t = 5.92, df = 52.71, p < 0.0001) than for females (1st year: 19.4 \pm 0.37 mm vs. 2nd year: 20.65 \pm 0.56 mm; t = 1.66, df = 104.83, p = 0.03). In the Central NY data set, females ranged from 10–35.5 mm and were larger than males (23.64 \pm 0.25 mm vs. 16.95 \pm 0.12 mm;

 $F_{1,1500}$ = 239.38, p < 0.0001). Allometric relationships between body size measurements (length, width of head capsule and mass) for insects in the Central NY data set are included in Suppl. material 1 (Suppl. material 1: Fig. S4).

Number of eggs per female ranged from 5 to 284. Number of eggs was positively related to body size and well described by a power function where number of eggs = $0.17 \cdot (\text{BodyLength})^{2.072}$ (Fig. 2). Analysis of residuals from the fitted power function showed no effect of tree species or voltinism on number of eggs ($F_{3,141} = 0.31$, p = 0.82). Egg lengths ranged from 0.99–1.51 mm with an average of 1.26 ± 0.01 mm. We found no difference in egg size between Spain and the USA (t = 0.48, df = 21.99,



Figure 1. Body size variation in *Sirex noctilio*. Females were larger than males and insects emerging from *Pinus resinosa* were larger than those emerging from *P. sylvestris* (Central NY and Northeast data sets plotted separately). Bars show mean \pm SE.



Figure 2. Relationship between body size and fecundity. Number of eggs (y) increases with female *Sirex noctilio* body size at the same rate for the main data set (closed circles) and Central NY data set (open circles). Line is fitted power function $y = 0.17x^{2.072}$ for the combined data set.

p = 0.64). There was some variation in egg size among wasps, but very little variation among eggs produced by each individual (estimated variation due to random effect in mixed model = 68 and 0%, respectively).

Host suitability and attack behavior

Dissection of 76 bolts from 24 trees (10 *P. radiata* and 14 *P. sylvestris*) yielded 11,253 attacks comprising 16,604 oviposition drill holes. Attack density was higher in *P. sylvestris* than in *P. resinosa* (8.83 \pm 1.29 vs. 5.28 \pm 1.72 attacks/dm²; $F_{1,51.72}$ = 4.43, p = 0.04). The number of drills per attack ranged from 1 to 6, distributed as follows: 64% were single drills, 27% doubles, 8% triples and 1% four or more. The number of drills per attack was slightly higher on average for *P. resinosa* (1.62 \pm 0.01) than for *P. sylvestris* (1.38 \pm 0.01) (Fig. 3; n = 11,253, z = -2.21, p = 0.03). The estimated variation in drill count per attack among bolts (nested within tree) and among trees (nested within site), was low (< 10% of the total random variation for each).

Emergence of adult wasps was concentrated in a relatively small number of bolts and was best described by a zero-inflated negative binomial (ZINB) distribution with parameters $\Phi = 0.24$, $\mu = 20.43$ and k = 0.85. For both tree species, emergence was — as expected — positively correlated (r = 0.36 - 0.79) with attacks, attack density, drills, drill density, drills per attack, estimated number of eggs laid and the estimated density of eggs laid, most of which were correlated with each other (Suppl. material 1: Table S1). In *P. sylvestris*, but not *P. resinosa*, variables associated with oviposition (estimated eggs laid, egg density), emergence per egg and total emergence were negatively correlated with bolt moisture, bluestain presence and lesion formation in response to attack (Suppl. material 1: Table S1). Lesion formation was positively correlated with bolt moisture level (r = 0.72) in *P. sylvestris* only. The percentage cross-sectional area of



Figure 3. Number of drills per attack across sampled tree species. *Sirex noctilio* attacks (oviposition sites) with 1, 2, 3 or 4+ drill holes for *Pinus sylvestris* and *P. resinosa*.



Figure 4. *Sirex noctilio* emergence success. Emergence per attack for *Pinus resinosa* (**A** slope = 0.08 ± 0.01) and *P. sylvestris* (**B** slope = 0.11 ± 0.01) and emergence per estimated number of eggs laid for *P. resinosa* (**C** slope = 0.16 ± 0.02) and *P. sylvestris* (**D** slope = 0.41 ± 0.04). Each point represents one bolt.

bluestain per bolt was positively correlated with both bolt moisture content and bolt volume (Suppl. material 1: Table S1).

Emergence per attack was higher for *P. sylvestris* (0.11 ± 0.01) than *P. resinosa* (0.08 ± 0.01) (Fig. 4); 63.8% of the *S. noctilio* produced came from the five trees that produced the most insects and these were all *P. sylvestris*. The proportion of emerging wasps that were male was higher for *P. sylvestris* (0.80) than for *P. resinosa* (0.64) (χ^2 = 28.60, df = 1, *p* < 0.0001). Insects emerging from *P. resinosa* were larger than those

emerging from *P. sylvestris* (Fig. 1; Northeast data set: $F_{1,32.98} = 4.95$, p = 0.03, Central NY data set: $F_{1,1500} = 29.61$, p < 0.0001) and the body size difference between males and females was more pronounced in *P. resinosa* than in *P. sylvestris* (Northeast only: $F_{1,974.24} = 8.85$, p = 0.003). Variation among trees within species accounted for 32% of the total random variation in adult size.

Species interactions

A total of 143 native *S. nigricornis* co-occurred with *S. noctilio* in our trees, but emergence of this native siricid was concentrated in three trees from one site (Table 2; Suppl. material 1). Hymenopteran parasitoids were abundant: we collected a total of 245 *Ibalia leucospoides* and 67 rhyssines (27 females and 40 males). Across all sites and years, total hymenopteran parasitism was $23.4 \pm 2.0\%$, with no difference between *P. resinosa* and *P. sylvestris. Ibalia leucospoides* parasitized $20 \pm 2.0\%$ of *S. noctilio* larvae, with no difference between tree species. The percentage of *S. noctilio* parasitized by *I. leucospoides* increased with total emergence in *P. resinosa* ($F_{1,11} = 5.11$, p = 0.04), but not in *P. sylvestris.* Rhyssine parasitism was $3.5 \pm 1.0\%$ overall and was higher in *P. resinosa* (9.22 $\pm 2.0\%$) than in *P. sylvestris* (1.72 $\pm 0.30\%$; $F_{1.34} = 15.57$, p < 0.001).

Of the 806 S. noctilio assessed for nematode parasitism, 62% contained nematodes in gonadal tissue, with no difference between sexes ($\chi^2 = 0.25$, df = 1, p = 0.62). We found no instances of nematodes within S. noctilio eggs. Wasps emerging in the first year were more likely to be parasitized (63.52%) than those emerging in year two (13.64%) (χ^2 = 22.64, df = 1, *p* < 0.0001). Among wasps emerging in year one, nonparasitized females were larger than parasitized females (22.03 ± 0.62 mm vs. 17.60 \pm 0.49 mm; t = 5.58, df = 158, p < 0.0001) with the same pattern seen in males (t = 4.75, df = 644, p < 0.0001). Females also had smaller eggs in the presence of nematodes (t = 3.17, df = 21.98, p = 0.004). Adult woodwasps that emerged from *P. sylvestris* were much more likely to be parasitized by D. siricidicola than those that emerged from *P. resinosa* (81.3% vs. 5.15%; χ^2 = 363.85, df = 1, *p* < 0.0001), but species and location were confounded (Table 2), so this finding could simply indicate spatial patchiness in nematode abundance. Estimates of nematode load per parasitized wasp ranged from 11 to 3,000 – the distribution of these values was best described by a negative binomial distribution with parameters $\mu = 395.2$ and k = 1.04. We found many wasps with < 500 nematodes and a few wasps with estimated parasite loads of up to ~ 2,000 or greater.

Reproductive potential

We constructed simple demographic models of *S. noctilio* in the northeastern USA, reporting study-wide values in addition to separate values for insects colonizing the two pine species in the study, *P. resinosa* and *P. sylvestris* (Table 3). An average-sized female of *S. noctilio* was predicted to contain 78 eggs (~ 66 eggs when emerging from *P. sylvestris* or 102 eggs when emerging from *P. resinosa*), based on body size-fecundity relationships. Nematodes did not enter *S. noctilio* eggs, so they had no direct impact on

Table 3. Demographic parameters for *Sirex noctilio* in Spain and the USA. Overall values for North America are shown, as well as separate values for *Pinus resinosa* and *P. sylvestris*. All data from Spain come from *P. pinaster*. Percent change in lambda ($\% \Delta \lambda$) is the percent change in USA λ when the Spain value for each row is substituted for the USA value. It indicates the relative impact of each of demographic factor on λ . Spanish values are adapted from Table 1 in Lombardero et al. (2016), with modifications to eggs/ Q^{-1} and emergences/egg² as described in Methods.

	Spain North America			North America by Species				
				P. resinosa		P. sylvestris		
	Parameter	Parameter	%Δλ	Parameter	%Δλ	Parameter	%Δλ	
Eggs/♀¹	58	78	-26	102	-43	66	-12	
Nematode escape	0.64	1	-36	1	-36	1	-36	
Emergence/viable egg ²	0.2	0.28	-28	0.16	27	0.41	-51	
Hymenopteran escape	0.8	0.77	4	0.77	4	0.77	4	
Proportion female	0.26	0.25	4	0.36	-28	0.2	30	
⊈/♀ (λ)	1.57	4.20	-63	4.52	-65	4.17	-62	

¹eggs/female calculated as the number of eggs expected in an average sized (20 mm) female; ²calculated, based on egg estimates in Madden (1974).

egg viability. Estimated emergence per egg was higher for *P. sylvestris* than for *P. resinosa* $(0.41 \pm 0.04 \text{ vs.} 0.16 \pm 0.02; \text{ Fig. 4})$, with an average of 0.28 ± 0.03 overall. From the combination of differences in fecundity and emergence per egg, we estimated 16 and 27 larval progeny per female adult for *P. resinosa* and *P. sylvestris*, respectively. A hymenopteran parasitism rate of 23.4%, consistent across tree species, further reduced estimated emergence to 13 and 21 adult progeny per female, of which 36% and 20% were female (Table 3). Thus, the estimated maximum number of females per female per year (λ) was about 4.2 for the overall population or 4.52 and 4.17 for *P. resinosa* and *P. sylvestris*, respectively.

Discussion

Life history traits

Non-native *S. noctilio* populations in North America showed a higher potential for population growth than native populations in Galicia, Spain (Table 3), with $\lambda > 4$ vs. 1.57. This was driven by the absence of sterilization by nematodes in North America and notable differences in fecundity and larval survival inside host trees. Despite this higher potential for population growth, trees with *S. noctilio* were rare in North America and stems with signs of *S. noctilio* attack often failed to produce adult progeny. Over 64% of *S. noctilio* in this study came from 13% of sampled trees, similar to findings from Foelker (2016), where 53.5% of *S. noctilio* emerged from 16% of sampled trees. Williams and Hajek (2017) and Haavik et al. (2018) also recorded wide variation in emergence and frequent failure of *S. noctilio* reproduction in attacked trees. Similarly, 32% of trees in this study had no insect emergence, compared to 41% in the native

range in Galicia, Spain (Lombardero et al. 2016). Despite increased sampling effort, Lombardero et al. (2016) collected only 313 adults from 134 trees compared to our 1007 from 38 trees.

Our results broaden the evidence of remarkably high body size variation for S. noctilio, consistent with reports of an 8–38 mm range in length from the native range (Lombardero et al. 2016). When compared to other insect species known to have high size variability, female S. noctilio showed roughly twice as much variation (over 4.1× versus 2.1× in the next most variable species; Suppl. material 1: Table S2). This is surprising given the clear link between body size and fecundity (Honěk 1993), although Madden and Coutts (1979) hypothesized that adult size variation is determined by nutrition and the success of the mutualist fungus A. areolatum and other recent studies have supported the hypothesis that highly variable environmental or nutritional factors help determine adult body size (Foelker and Hofstetter 2014; González et al. 2014). This is also consistent with recent findings by Garnas et al. (2020), where a complex interplay of factors related to resource quality influenced woodwasp body size, even within a single tree. Although P. resinosa produced fewer S. noctilio individuals, they were ~ 23% larger than those produced in *P. sylvestris*, suggesting that *P. resinosa* is an attractive host, but may have higher host resistance to S. noctilio. When oviposition is successful, P. resinosa provides a suitable and perhaps superior substrate for A. areolatum growth and S. noctilio maturation.

High variation in woodwasp body size could also reflect genetic differences among wasps and complex selective landscapes that favor large insects in some instances, but not in others. Long feeding galleries necessary to produce large adults may be more feasible in large trees than small trees. Large males may have difficulties mating with small females (Caetano and Hajek 2017) and large adults of either sex may be more vulnerable to predators. The large size of female *S. noctilio* relative to males suggests the effect of natural selection on female size (likely favoring higher fecundity) is considerably stronger than natural or sexual selection on males (Wiklund and Karlsson 1988).

The fecundity of North American *S. noctilio* is higher than in Spain, but this is not driven by differences in body size. An average-sized female in our sample (19.67 mm long) had ~ 78 eggs. Although Spanish *S. noctilio*, measured by Lombardero et al. (2016), had a similar range in egg number (0 to 270 eggs per female), 19.7-mm females in Galicia, Spain were estimated to have ~ 56 eggs. Other North American studies also show elevated *S. noctilio* fecundity compared to native populations (Table 1). On the other hand, fecundity in this study was lower than that reported for Southern Hemisphere populations (e.g., 50–500 in New Zealand; Zondag and Nuttall 1977). The higher fecundity of *S. noctilio* in the Southern Hemisphere may be attributable to larger average body size, which has been widely reported (e.g., 24–32 mm average body size for females in South Africa; Hurley et al. 2008).

At a population level, observed male-biased sex ratios were similar to those previously reported in North America (see Table 1) and in the native range in Galicia, Spain (Lombardero et al. 2016). In this study, spatial patterns in North American sex ratios were opposite of what would be expected if the male bias were due to low mating success at the range edge since the lowest proportion of males was in the most recently invaded region (Blacklick in central Pennsylvania) where attack densities were low. However, sex ratio has been found to vary widely among sites in other studies (e.g., Haavik et al. 2018) and has the potential to influence local or regional outbreak patterns.

Host selection and resistance

Host choice and oviposition behavior influence the success of *S. noctilio* in new landscapes. In our study, 64% of total attacks involved only a single drill. Single drills are thought to indicate rejection of the oviposition site (since eggs are rarely placed), perhaps as a consequence of the detection of suboptimal moisture levels and/or oleoresin pressure for fungal establishment, egg survival or larval success (Ryan and Hurley 2012; Hayes et al. 2015). Attacks with two or more drills normally contain eggs, as well as fungi and venom (Madden 1974). In Spain, only 43% of total attacks involved a single drill and, in Argentina, only 39% had one drill (Martinson et al. 2018). Evidently, the US stands in this study included a higher proportion of trees that were judged as low quality by ovipositing females. This may be due, in part, to rejection of trees with higher moisture content (Suppl. material 1: Table S1), variable host species attractiveness and differences in biotic and abiotic drivers of tree stress in different regions (Madden 1988; Haavik et al. 2017; Corley et al. 2018). Any factors that reduce host attractiveness and, thus, oviposition by female woodwasps have the potential to suppress population growth that can lead to outbreaks.

The fate of larvae inside host trees strongly influenced reproductive potential in both US and Spanish S. noctilio populations. This is consistent with past studies that have highlighted the role of host resistance in limiting S. noctilio reproduction and spread (Haavik et al. 2017; Haavik et al. 2018). Mortality at the egg and larval stages consistently reduced numbers of female offspring by over 70% (Table 3, Lombardero et al. 2016) and our finding of much higher reproductive success in P. sylvestris than in *P. resinosa* further highlights the potential for tree-specific factors to heavily influence S. noctilio population dynamics. However, other demographic factors also matter. For example, the lower survival of larvae feeding in P. resinosa vs. P. sylvestris was almost completely compensated by larger adult size (and, therefore, higher fecundity) and a higher proportion of females (Table 3). In the northeastern United States, small localized outbreaks have occurred, but tended to decline after several years as susceptible host trees were depleted. The potential for woodwasp population growth has probably been highly constrained in the Northeast by the limited availability of suppressed trees within stands of hard pines which are themselves rare in the landscape (Haavik et al. 2016).

Top-down controls on S. noctilio

Spanish woodwasp populations experience consistent top-down control by nematodes via sterilization of ~ 90% of the eggs in 39% of females (Lombardero et al. 2016). In contrast, sterilization of eggs by parasitic nematodes was absent in our US study popu-

lations. Although we found nematodes in ~ 63% of wasps sampled, they were only present in the body cavity, not within the eggs. Previous North American studies have also reported that *D. siricidicola* fail to sterilize *S. noctilio* eggs (but see Kroll et al. 2013 and Williams and Hajek 2017 for minor exceptions). Nematode impacts on reproductive potential in the region are, therefore, indirect, but may still be consequential: parasitized females in North America were smaller and less fecund than unparasitized females (17.6 mm and ~ 65 eggs vs. 22.0 mm and ~ 103 eggs). If nematode parasitism reduces adult body size, this corresponds to a 37% drop in the reproductive potential (λ) for parasitized vs. unparasitized females.

Our finding that nematode parasitism dropped to under 14% for larvae that took two years to develop supports the hypothesis that delayed development can help woodwasps evade parasitism (Corley and Bruzzone 2009). The incidence of nematode parasitism was also clumped (better fit by a negative binomial than a Poisson distribution; Vale et al. 2013). Presumably the patchy dispersion of nematodes reflects variation among trees in the introduction of nematodes by ovipositing woodwasps, in the establishment of *A. areolatum* on which the nematodes feed, and perhaps in the relative timing of wasp and nematode development, which must be synchronized for nematodes to effectively disperse inside emerging woodwasp adults. Even in Southern Hemisphere plantations where *D. siricidicola* is deployed for biological control, parasitism rates vary widely (Slippers et al. 2012) and annual augmentative releases are regularly practiced and typically necessary for effective control.

Hymenopteran parasitoids exerted a moderate top-down influence on North America S. noctilio, reducing the number of progeny by ~ 20%, which is within the range of reports from other studies in North America (~ 1-50%; Table 1) and the native range. In the most robust study of S. noctilio parasitoid complexes in North America to date, Foelker et al. (2016) found similar overall rates of hymenopteran parasitism (27.6% and 20.9% in 2010 and 2011). We found evidence of density-dependent effects from I. leucospoides at the tree level in P. resinosa. Similarly, Haavik et al. (2016b) found that *I. leucospoides* presence in Ontario was positively correlated with the density of attacked pines within stands. Ibalia leucospoides uses olfactory cues to locate S. noctilio oviposition sites, based on the presence of volatiles from the woodwasp mutualist A. areolatum (Martínez et al. 2006) and may detect resource-rich patches from some distance (Fischbein et al. 2012). A Type III functional response to S. noctilio presence has also been reported for hymenopteran parasitoids in Argentina, with attack rate increasing as the availability of S. noctilio oviposition sites increases (Fernández-Arhex and Corley 2005). At high S. noctilio densities, this parasitoid can help to suppress outbreaks. However, even if our highest I. leucospoides parasitism rate (33%) was found across all sites, this would only decrease lambda for S. noctilio in the northeastern USA by ~ 17% (Table 3). We suspect that the extreme patchiness of *S. noctilio* resources in the current North American range, combined with the solitary lifestyle and life history of *I. leucospoides* (Haavik et al. 2015), limits the potential for population regulation by these parasitoids.

Future S. noctilio expansion in North America

The presence of native pines, native siricids and native siricid parasitoids in North America may confer some biotic resistance to invasion by S. noctilio (Foelker 2016; Nunez-Mir et al. 2017). This differs from the Southern Hemisphere where neither pines nor their associates are native. Despite the similarities to the native range, our demographic analysis suggests higher population growth potential in North America vs. Spain (Table 3). This may result in complex dynamics if the woodwasp reaches native pine forests that are overstocked or experience frequent drought stress, attack by other pests and pathogens or wildfire (Adams et al. 2010; Anderegg et al. 2015). Interactions with other insects will also help shape these dynamics. Our study did not attempt to quantify competition, but we did see reduced S. noctilio emergence in the presence of bluestain fungi associated with bark beetle colonization. This is consistent with other studies where co-occurrence with other subcortical insects has been shown to reduce the reproductive success of S. noctilio, likely mediated by competition among fungal associates (Ryan et al. 2012). These fungal interactions also have the potential to influence woodwaspnematode interactions and were found to reduce the survival of *B. siricidicola* and, thus, the effectiveness of biological control efforts, in Australia (Yousuf et al. 2014).

All hard pine species in North America are potentially susceptible to *S. noctilio* (Dodds and de Groot 2012). Populations in Michigan and southern Ontario colonize *P. banksiana* and will continue to encounter this host as they spread into new regions. Woodwasps expanding southwards will soon encounter southern pines including *P. palustris*, *P. taeda* and *P. elliottii* and establishment in western North America would permit colonization of lodgepole pine, *P. contorta*, which has been shown to be highly susceptible to outbreaks in Argentine Patagonia (Lantschner and Corley 2015). More comprehensive knowledge of variation among pine species in susceptibility to *S. noctilio* would help managers anticipate future impacts in North America.

More broadly, a comprehensive comparison of the population demography of Northern and Southern Hemisphere *S. noctilio* would help us better elucidate the importance of specific controls on *S. noctilio* populations and improve understanding of variable tree species susceptibility. Such studies would also help clarify the importance of landscape-level patterns in resource availability in determining woodwasp population growth rates. Current and future population models would also be improved by better understanding and incorporating density-dependent feedbacks in the demographics.

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Supplementary material I

Tables S1, S2, Figures S1–S4

Authors: Flora E. Krivak-Tetley, Jenna Sullivan-Stack, Jeff R. Garnas, Kelley E. Zylstra, Lars-Olaf Höger, María J. Lombardero, Andrew M. Liebhold, Matthew P. Ayres Data type: Text, tables and figures (docx. file)

- Explanation note: This file includes demographic data from native *Sirex nigricornis* woodwasps that were collected during our study. Table S1. Correlation matrix of bolt-level variables for *P. resinosa* and *P. sylvestris*. Table S2. Review of 10th and 90th percentile female body mass from insect species estimated from source paper cited, showing comparatively higher female *S. noctilio* body size variation. Figure S1. Examples of *S. noctilio* oviposition sites, lesion formation and emergence holes in the field. Figure S2. Top panels show a typical bolt from *P. sylvestris* with bark removed and *S. noctilio* emergence holes and oviposition sites identified. Figure S3. The number of *S. noctilio* emerging per tree was best described by a zero inflated negative binomial distribution (dotted line) with proportion of excess zeroes $\Phi = 0.32$, μ = 75.39 and overdispersion parameter k = 0.35. Figure S4. Allometric relationships for *S. noctilio* in the Central NY data set: number of eggs and adult female mass (a; quantile regression), adult female mass and adult female length (b; fitted power function) and adult length and width of adult head capsule (c; linear regression).
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RESEARCH ARTICLE



Invasive catfish in northern Italy and their impacts on waterbirds

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Abstract

Predatory fish have occasionally been observed preying on birds, sometimes repeatedly, but few studies were able to unravel the overall significance of avian prey in fish diet and the predation impacts on bird populations. We used a control/impact study setup, using a Nature Reserve in northern Italy and a nearby control area, to determine: 1) the contribution of waterbirds to wels catfish diet in the Reserve, 2) the population density of wels catfish in the Reserve and control area and 3) the potential impacts of waterbird depredation by wels catfish on waterbird population trends. Our stable isotope Bayesian mixing model indicated that birds contributed 12.2% (5-27.9%, 50% confidence interval) of the diet of large wels catfish (> 98 cm in total length). Large individuals constituted the majority of the population in the shoreline areas of the reserve in 2013–2019, where the population was stable despite control efforts. Numbers were below detectable levels in the control area. Large wels catfish consumed an average of 224, 148 and 187 kg of birds during the 2019 chick growing period, as estimated through three different bioenergetic models. Compared to the control area, mallard reproductive success was diminished in the Reserve, likely due to higher rates of fish predation, although effects were variable in different years. Overall, our data suggest that high densities of invasive wels catfish might impact waterbird reproductive success through predation on bird chicks, but further studies would be needed to reduce uncertainties related to the intrinsic variability of field ecology data. Our study constitutes a preliminary attempt to assess the potential of introduced wels catfish to affect the conservation value of waterbird protection areas, and should be repeated at broader spatial and temporal scales.

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Keywords

Predation, pulsed resources, Silurus glanis, stable isotopes, waterbirds, wels catfish

Introduction

Wetland loss has been particularly severe in the Mediterranean Region, where habitat conservation is still at lower levels than in other areas of the world (Hoekstra et al. 2005; Leberger et al. 2020), highlighting the need to maximise the effectiveness of existing protection, especially in Italy (Maiorano et al. 2007). The limited ability of habitat protection to address and control other threats affecting species distributions and biodiversity (e.g. climate change, pollution, biological invasions) could be at the root of differences in protection performance between areas (Pavón-Jordán et al. 2015; Gaget et al. 2020). Invasive species often diminish the effectiveness of protected areas, particularly those aimed at birds nesting on islands, with a wide range of cascading effects (Fukami et al. 2006). Invasive aquatic species have also been highlighted as a threat to freshwater protected areas (Saunders et al. 2002). Invasive fish have been considered as particularly detrimental for amphibians via predation (Vredenburg 2004; Finlay and Vredenburg 2007; Pope 2008) and for birds via competition and habitat modification, especially invasive cyprinids (Bajer et al. 2009; Epanchin et al. 2010; Maceda-Veiga et al. 2017). Waterbirds are flagships for wetland protection (e.g. under the Ramsar convention) and invasive terrestrial predators, such as mammals, have often been controlled to improve the breeding success of waterbirds (Gilbert et al. 1996; Whitehead et al. 2008). However, as yet, little consideration has been given to the potential impact of invasive predatory fish on birds.

Predatory fish have been occasionally observed preying and sometimes focusing, on birds. In marine environments, the giant trevally (Caranx ignobilis) has been observed hunting sooty terns (Onychoprion fuscatus) in the Seychelles (Attenborough 2017). A species well-known for its dietary breadth, the tiger shark (Galeocerdo cuvier) has been found to prey on terrestrial birds (rather than seabirds) along the coast of Alabama, perhaps exploiting extreme weather events that force migrating birds to land on water (Drymon et al. 2019). Large freshwater predatory fish like the taimen (Hucho taimen) (Kottelat and Freyhof 2007), the murray cod (Maccullochella peelii) (Harris and Rowland 1996), the northern pike (Esox lucius) and the muskie (Esox masquinongy) can also hunt birds. Northern pike presence has been shown to drive the habitat choice and reproductive success of nesting ducks through predation, especially on ducklings (Dessborn et al. 2011), although this effect was not clear in earlier studies (Lagler 1956). In this context, stomach content analysis is unlikely to yield a significant answer, as it integrates fish diet over a limited timespan (Windell 1968) and would require extremely intensive field sampling to detect occasional prey, such as birds. Stable isotopes, on the other hand, have been routinely used to investigate diet, as they integrate information over a



Figure 1. Location of the study area in Italy (upper left panel, in red) and location of the control (in green) and test (in orange) areas at the opposite ends of Lake Iseo (upper right panel). The lower left panel depicts the control area (in green), where the Oglio River enters Lake Iseo. The lower right panel depicts the test area, the Sebino Peat Bogs (*Torbiere del Sebino*), a Nature Reserve declared in 1984. Three different areas of the Reserve are named and shaded in yellow, blue and magenta, based on their depth, vegetation and numbers of human activities permitted. Satellite and aerial imagery are from Google Earth.

longer timespan (Perga and Gerdeaux 2005) without needing extensive sampling. Wels catfish (*Silurus glanis*) has been reported to feed on birds (Carol et al. 2009; Copp et al. 2009; Syväranta et al. 2010) and its large size makes it capable of preying on adults of large species, such as great cormorants (*Phalacrocorax carbo*, this study). Cucherousset et al. (2012) suggested that wels catfish are able to learn complex predation strategies, which were used to hunt bathing pigeons and showed that birds could reach 30–40% of the diet of specialised individual fish. However,

only one study attempted to unravel the overall significance of birds in wels catfish diet (Cucherousset et al. 2018) and its potential impacts on bird populations remains poorly understood.

Wels catfish is native to eastern Europe and western Asia and has been widely introduced to western Europe, where it quickly became invasive, but its impact on invaded ecosystems is still not fully understood (Copp et al. 2009). In Italy, wels catfish is now widespread, especially in the northern part of the country (Po River Basin). It grows faster than in its native range (Rossi et al. 1991) and has been highlighted as a potential driver of native fish decline (Castaldelli et al. 2013). We used a control/impact study setup, using a Nature Reserve in northern Italy and a nearby control area, to determine: 1) the contribution of waterbirds to wels catfish diet in the reserve, 2) the population density of wels catfish in the Reserve and control areas and 3) the potential impacts of waterbird depredation by wels catfish on waterbird population sustainability. We hypothesised that invasive wels catfish predation could be a potentially significant pressure on waterbirds, especially during the nesting season and on chicks. We assumed predation would be predator-density dependent and expected predation to be size-limited, so that young birds would be the main prey and larger wels catfish to have a higher proportion of birds in their diet. Ultimately, our study constitutes a preliminary attempt to assess the potential of introduced wels catfish to affect the conservation value of waterbird protection areas.

Materials and methods

Study setup

To assess the effects of wels catfish predation on birds, we utilised a control/impact approach, assuming that predation would be density-dependent (i.e. that it would be less significant in the control area, where predator density is much lower). We used a preliminary analysis of wels catfish stomach contents to guide our field sampling of their putative prey items. We then estimated the avian contribution to the diet of wels catfish in the Reserve using a stable isotope Bayesian mixing model and its biomass using electrofishing removal. Based on three different bioenergetic models, we estimated wels catfish daily feeding rates and used this information to estimate birds' consumption by wels catfish in the Reserve (impact area) during the nesting and chick growing period (April-June, 90 days) of 2019. We then used mallard (*Anas platyrhynchos*) chick counts in 2017–2019 to compare reproductive success in the control and test areas, to gauge potential differences in predation magnitude and considered reproductive bird trends in the Reserve and surrounding areas to detect any broad effects.

Study area

Our control area consisted in the area where the Oglio River flows into Lake Iseo, about 17.5 km north of the Reserve. Both control and test areas share an equal number

and type of other potential predators (e.g. birds or mammals), but the control area is characterised by high anthropogenic presence, no bird protection measures (i.e. hunting is allowed) and slightly deeper, flowing water. Wels catfish is present in the control area, but at much lower densities than in our test area (this study).

Our test area consisted in the Sebino peat bogs ('Torbiere del Sebino', in Italian), a marshland of ~ 360 ha, located near the southern shore of Lake Iseo (6530 ha), in northern Italy. These bogs are typically shallow (average depth 5 m, this study) and coldtemperate (5.8-28.3 °C during 2019, this study), with abundant emergent and submerged aquatic vegetation and are intermittently connected to the Lake. The Lamette part of the bogs is a shallow (max. depth 5 m) marshland with abundant reeds that has the closest connection with the Lake and is a strict Reserve (i.e. no human activities are allowed). Conversely, the 'Torbiere' and 'Lama' parts of the bogs are a series of deeper (max. depth 9 m) lakes, have restricted public access and, in some parts, recreational fishing is permitted (Fig. 1). The Sebino Peat Bog Nature Reserve (hereafter referred to simply as the Reserve) was established in 1984 and is protected under both national and European legislation (Natura 2000 network). Prior to this protection, the bogs were used extensively for peat harvest. The Reserve is a key nesting area for waterbirds, as well as an important stopover area during the migration period and a relevant wintering site (Trotti 2019). Out of the total 268 bird species observed in the Reserve since the 1950s, 14 are introduced and 31 are covered by the EU Birds Directive (Trotti 2019).

Wels catfish were first accidentally introduced to Lake Iseo through the Oglio River and ultimately to the Reserve in the late 1980s (Mancini, unpublished data), but their numbers in the Reserve were initially low and they were not detected until much later. Wels catfish biomass in the Reserve is likely to have increased around 2005–2008, leading first to their detection and then to selective harvest in 2011, to limit the introduced fish population in the Reserve (Mancini, unpublished data).

Fish diet analysis

We assumed that small-sized catfish would not be effective predators of adult and subadult birds, due to mouth gape limitations. Therefore, we sampled 31 large-sized wels catfish (total length > 98 cm, hereafter simply referred to as length) by spearfishing in the Reserve during spring-summer 2019. These individuals were analysed for stomach contents using a volumetric point method (Windell 1968) to collect preliminary information on catfish diet and guide sampling of putative prey for stable isotope analysis. Not unexpectedly for predator fish, 20 wels catfish stomachs were found empty and 11 stomachs contained prey, of which four contained red swamp crayfish (39.1% of total combined volumetric contents), three contained unidentified remains (13%), two contained fish of different sizes (Italian rudd, *Scardinius hesperidicus* and unidentified, 13%), one contained a rodent (unidentified, 8.7%), one a feather and one an adult bird (cormorant, *Phalacrocorax* spp., 26.1%).

Based on this guidance and on literature dietary information (e.g. Copp et al. 2009), we sampled catfish muscle from our preliminary sample and a range of putative

prey species from the Reserve for stable isotope dietary analysis. Permits for collection of field samples were obtained from the 'Torbiere del Sebino' Nature Reserve administration within the wels catfish control programme, all individuals were immediately euthanised after capture and no protected species were culled in this study. We collected muscle of putative aquatic prey, including large piscivorous fish (three individual samples from two species), small generalist fish (12 individual samples from six species) and alien red swamp crayfish (*Procambarus clarkii*, eight individual samples) through electrofishing. We also collected muscle from putative terrestrial prey, including one rodent muscle (*Rattus* sp. from fish stomach contents) and feather (calamus) samples from several waterbird species (43 individual samples from eight species, from fish stomach contents, birds found dead and natural moulting) through an opportunistic collection. A detailed list of samples and species is provided in Suppl. material 1: Table S1. Collecting feathers allowed for non-lethal sampling of birds, while offering a stable isotope measure comparable to muscle tissue (Hobson and Clark 1992a, b).

Tissue samples were dried at 60 °C, ground to fine powder (muscle) or cut to size (feathers). As lipid variations in tissues can alter d¹³C values, feathers were rinsed in 2:1 chloroform/methanol solution to remove surface lipids and stable isotopes ratios of C in other tissues were later arithmetically corrected for lipid content (Kiljunen et al. 2006). Samples were analysed for stable isotope ratios of C and N at the University of Jyväskylä, Finland, using a Thermo Finnigan DELTA^{plus} Advantage continuous-flow stable isotope-ratio mass spectrometer (CF-SIRMS), coupled with a FlashEA 1112 elemental analyser (Thermo Electron Corporation, Waltham, MA, USA). The resulting ratios were expressed in terms of relative concentrations relative to a laboratory reference standard. While a rodent and a feather sample collected from stomach contents were isotopically analysed, they were eventually excluded from further analysis to avoid issues with potential consumer contamination and low sample size.

We estimated diet proportions of wels catfish with a Bayesian mixing model under R statistical software 3.6 (R Core Team 2019) through the mixSIAR package (Stock et al. 2018), which accounts for sample size when estimating diet proportions and using literature isotope fractionation [δ^{13} C = 0.4 ± 1.2, δ^{15} N = 2.3 ± 1.6, (McCutchan et al. 2003)]. We tested for wels catfish size effects by running a mixing model where catfish length was considered as a continuous covariate, after testing that differences in diet composition between size classes suggested by our preliminary stomach content analysis were not significant in the isotopic space (PERMANOVA P-value > 0.05). We also used our preliminary stomach content analysis and expert judgement to produce informative priors (see Suppl. material 1: Fig. S3) that were used in the mixing model (Moore and Semmens 2008), with a very conservative approach to bird consumption values. We additionally ran re-sample simulations (100 iterations each for samples sizes 2-50) to assess isotopic sources sample size effects on the results of the model, using the package 'samplesim' (Casajus et al. 2021). Putative prey species were divided into five functional groups (Small fish, Large Fish, Crayfish, Invertivorous Birds, Herbivorous Birds and Piscivorous Birds), based on taxonomy and ecology. The piscivorous birds included cormorants and grebes (thus with both a marine and freshwater signal),

the herbivorous birds included swans, mallards, pochards and moorhens and the invertivorous birds included water rails. Chicks of most waterbird species are invertivorous during growth (Sugden 1973), but no specific groups were created for chicks.

Fish biomass estimation

To estimate wels catfish biomass in the Reserve, we used 73 boat-mounted electrofishing events between 2013 and 2019, covering the shoreline of all three main areas of the Reserve ('Lama', 40 events, 'Lamette', 23 events, 'Torbiere', 10 events) and all seasons (but focusing on autumn and spring). A total of 1356 individuals were caught and removed from the Reserve using electrofishing, for an overall weight of 8113 kg. Wels catfish were of average total length 84.45 cm (median 82 cm, min. 2.2/max. 211 cm) and of average weight 5.98 kg (median 3.84 kg, min. 0.015/max. 92.75 kg).

We estimated the overall biomass of wels catfish in each of three areas of the Reserve, by averaging the detected density at each sampling event. Density was calculated as a function of biomass harvested and area sampled during each event, where area was the length of the shoreline fished, multiplied by the effective radius of the electrofisher (i.e. 5 m). Density trends over time were analysed with linear regressions. Given that we only estimated density in shoreline areas and that electrofishing catchability is high but not perfect, ours was likely an underestimation of total biomass.

To estimate wels catfish biomass in the control area, we used a boat-mounted electrofishing survey followed by three visual census surveys, carried out between 2012 and 2016 and spanning from April to July, along the shorelines of the control area (including the terminal part of the Oglio River).

Wels catfish consumption models

We estimated wels catfish annual consumption of prey by developing a specific bioenergetic model for the local conditions and sampled size cohorts. We used the Wisconsin bioenergetic model (Hanson et al. 1997), parametrised according to experimental studies on wels catfish and similar species (Hilge 1985; Raat 1990; Xiao-Jun and Ruyung 1992; Bourret et al. 2008, see Suppl. material 1: Table S2) to estimate annual consumption. Fish being poikilotherms, the model relies on water temperature to assess metabolic rates and on prey energy content to model body mass gain. We thus used records of daily average temperatures in the Reserve in 2019 (when catfish were sampled, see Suppl. material 1: Fig. S1), food energy contents from Cummins and Wuycheck (1971) (see Suppl. material 1: Table S3) and diet composition derived from our stable isotope analysis as inputs to the model. We used model results to calculate an average daily consumption of wels catfish during the chick growing period, accounting for site-specific diet composition, size and growth (see Suppl. material 1: Table S3), estimated based on yearly length increments from Rossi et al. (1991). Weight increments were derived from length, using a weight/length regression fitted to our data $(W = 7E-05*L^{2.6535}, R^2 = 0.9374).$

Additionally, we compared our model results with two previous consumption estimates. An average daily consumption of 1.99% of wet mass day⁻¹ was estimated by Omarov and Popova (1985) and Orlova and Popova (1986) for wels catfish in Georgia over the whole year. A similar value of 1.32% of wet mass day⁻¹ was also estimated by Copp et al. (2009) in their review, also including European data.

We then used the estimated biomass and the three different estimates of average daily consumptions listed above to calculate the total quantity (kg) of prey ingested over a year. The consumption of bird prey was then estimated, based on its dietary proportion, as estimated by our stable isotope mixing models, accounting for the error in the dietary contribution (50% confidence interval) and in the biomass estimate (SD around the mean) when calculating the upper and lower confidence limits of the consumption estimate. Bird consumption was estimated exclusively for the > 98 cm size cohort, as diet was determined only for this size class.

Bird abundance and trends

We used nesting bird surveys from 2002–2019 (Brichetti 2002; Longo 2009; Trotti et al. 2016; Trotti 2017, 2018, 2019) to assess the consistency and short-term trends of waterbirds (i.e. ecologically dependent upon wetlands) nesting in the Reserve, which could be potential prey for wels catfish. Surveys were carried out by 1-3 experienced observers, between May and September, for a total of more than 30 days per year. Surveys included nestling and chick counts during and after the breeding season and were performed from both shore and boats, at dawn and dusk. We focused on nesting birds, as young birds are smaller and likely to be more readily predated and the breeding season coincides with the period of warmer temperatures, which increase fish activity and thus predation. Given the few data points, trends in nesting birds numbers were identified using simple linear regressions and given a qualitative classification as Stable (coefficient <0.2), Moderate increase/decrease (0.2 < coefficient <0.5) and Increase/Decrease (0.5 < coefficient), reporting only R² values. Additionally, longterm trends in wintering numbers estimated by the International Wetland Census for the functional/ecological spatial wintering unit that includes the study area, plus contiguous spatial units (Zenatello et al. 2014) were also retrieved for comparison of local and larger spatial scale trends.

To offer a comparison of the scale and potential impact of wels catfish consumption on birds, we used average weights of each bird species (accounting for sexual dimorphism in size, see Suppl. material 1: Table S4) to estimate an overall nesting adult biomass in the Reserve during the summer season (i.e. the season when fish predation is most likely). We may have underestimated the total waterbird biomass because we did not include the weight of chicks and there may be constant turnover of individuals in the bird population. Furthermore, the waterbird biomass would be considerably higher in winter, when bird numbers increase.

Finally, we used reproductive success surveys, carried out in 2017–2019 with similar methods as the nesting bird surveys, to assess differences in the number of chicks



Figure 2. Isotopic space positions of wels catfish and its putative prey sources in the Reserve, corrected for isotopic fractionation. Error bars represent standard deviations of each prey source. Feathers were analysed for birds and muscle for other taxa; both were corrected for lipid content.

per couple of waterbirds in the control and the Reserve areas. We chose mallard as a test species, as it is a cosmopolitan and abundant species in both areas and counted the number of chicks per couple in early (mid-April/mid-May) and late (June) stages of the chick growing period in order to test differences in the median number of chicks at the late stage across the areas and differences in the slopes between early and late stages across areas, under the null hypotheses that different areas would have equal means and slopes. We used the non-parametric Mann-Whitney test on the medians and the test on the difference between the slopes from two independent samples outlined in Howell (2012).

Results

Fish diet

Wels catfish in our sample (n = 30, mean 139.9 cm, SD 30.5 cm, length range 98-191.5 cm) were generally spread between sources in isotopic space (Fig. 2), but there were no clear trends stemming from wels catfish length in the mixing model.

The Bayesian mixing model indicated that birds composed 12.2% (5–27.9%, 50% confidence interval) of the diet of wels catfish > 98 cm in length. More specifically, invertivorous bird prey composed 8.6% (4.6–14.1%, 50% confidence interval) of the diet, while herbivorous and piscivorous bird prey composed 1.7% (0.2–6.4%, 50% confidence interval) and 1.9% (0.2–7.4%, 50% confidence interval) of the diet, re-

spectively (Fig. 3). According to the mixing model, larger catfish did not have a higher proportion of birds in their diet.

Our sample size simulation indicated that increasing sample size up to 50 samples was likely to downplay the dietary proportions of crayfish and large fish and increase the relevance of birds (particularly piscivorous birds) by up to ~ 5%, but not to decrease the width of confidence intervals, except for invertivorous birds (see Suppl. material 1: Fig. S4a, b). Doubling wels catfish sample size would have similarly yielded a ~ 10% increase in the relevance of birds (particularly invertivorous birds) and a similar decrease of the width of confidence intervals (see Suppl. material 1: Fig. S4c).

Fish biomass

Detected wels catfish density ranged 3.4-174 kg/ha, with highest densities recorded in the 'Lama' part of the Reserve and no clear trends were observed in 2013–2019 (see Suppl. material 1: Fig. S2). The estimated average total biomass of wels catfish (of all sizes) along the Reserve shorelines was 1237 (\pm 254) kg. Despite being only 22.9% of the total individuals sampled, fish 98–191 cm of length were estimated to have an average biomass of 1024 kg, 83% of the total biomass.



Figure 3. Curves of dietary proportions of wels catfish prey sources, derived from the Bayesian mixing model for stable isotopes including weakly informative dietary priors.

None of the surveys in the control area was able to detect wels catfish, despite covering a total combined surface of 75.7 km². We thus conservatively concluded that, albeit present in Lake Iseo, wels catfish density in the control area was below detectable levels and, thus, likely to be negligible compared to the biomass detected in the Reserve.

Bird consumption

Our bioenergetic model suggested an average daily ratio of 1.7% wet mass day⁻¹ for a wels catfish > 98 cm during the chick growing period (and an average daily ratio of 1.5% wet mass day⁻¹ over the whole year).

The estimated average bird consumption for the wels catfish population > 98 cm during the chick growing period was 224, 148 and 187 kg, respectively, as estimated through the three different daily ratios (Fig. 4). By comparison, the overall biomass of the 243 waterbird nesting pairs in the Reserve during 2019 was estimated at 792 kg (691 kg excluding adult swans, Fig. 4).



Figure 4. Estimated consumption of birds by wels catfish in the shoreline of the Nature Reserve during the chick growing period of 2019, as obtained with the three different estimates of daily consumption ratios. Error bars account for uncertainty in both dietary and biomass estimations. The horizontal dashed lines indicate the estimated biomass of nesting adult waterbirds observed in the Reserve during the chick growing period of 2019.



Figure 5. Mallard chicks per pair in the control (green) and test (orange) areas, as detected in early (mid-April/mid-May) and late (June) stages of the chicks growing period. In boxplots, black horizontal lines represent medians, boxes represent the first and third quartile, whiskers represent minimum and maximum values and dots represent extreme values.

Bird population trends

A total of 12 waterbird species that could be potential prey of wels catfish were found nesting in the Reserve in 2002–2019, for a total of 243 nesting pairs. The most abundant breeding species was the great cormorant (*Phalacrocorax carbo*, 83 breeding pairs), while the least abundant was the mute swan (*Cygnus olor*, five breeding pairs). The number of breeding pairs was generally consistent in 2017–2019 for most species (Table 1), increasing for some species (e.g. the great cormorant), but markedly lower than those detected in 2002 for other species (e.g. Eurasian coot, *Fulica atra*). The total estimated nesting waterbird biomass in 2019 was 792 kg (691 kg, excluding adult swans which are particularly large prey).

During 2017–2019, mallard reproductive success at early stages of the chick growing period was equal in the test rather than in the control area (Fig. 5, Mann-Whitney P-value > 0.7 for all years). However, in 2018 and 2019, there were statistically significant differences between the test and control areas in the number of chicks at late stages of the growing period (Mann-Whitney P-values < 0.01 and < 0.05, respectively), even if those were not significant in 2017 (Mann-Whitney P-value = 0.06). The differences in slopes expressing the rate of decline were statistically significant in 2018 (P-value < 0.03), but not in 2017 and 2019 (P values 0.3 and 0.06, respectively).

Discussion

Regarding objective 1, our analysis confirmed that the diet of the wels catfish population in the Reserve included birds, albeit their median diet proportion (12.2%) was not as high as in a previous study that focused on specialised individuals (Cucherousset et al. 2012). As for objective 2, we found the shoreline areas of the Reserve hosted a population composed mainly of large (> 98 cm) wels catfish and its biomass (1024 kg) was stable in 2013–2019 despite population control efforts, while wels catfish biomass in the control area was below detectable levels. For objective 3, we found that these fish consumed a relatively small portion of birds during the chick growing period, as estimated through different daily ratios using the median diet proportion of birds. According to our data (Table 1), for some of the species of waterbirds exposed to fish predation when breeding, the number of nesting couples declined after wels catfish was detected in the Reserve and stabilised to lower levels after that. Additionally, compared to the control area, mallard reproductive success was diminished in the Reserve, even if effects were variable in different years. This appears to be the case for precocial birds, such as waterfowl, coots and grebes that rear their young on the water, but not for altricial herons and cormorants which rear their young in the nest (Table 1). Overall, our data suggest that high densities of invasive wels catfish might impact waterbird reproductive success through predation on bird chicks, but further studies would be needed to reduce uncertainties related to the intrinsic limitations of field ecology. Ultimately, our study constitutes a preliminary attempt to assess the potential of introduced wels catfish to affect the conservation value of waterbird protection areas and further investigation is required.

Fish diet composition is often driven by prey availability, while fish dietary intake is a function of activity and metabolism, which are mostly driven by body size and temperature, so uncertainties might be compounded in the final estimate of predation effects. Cucherousset et al. (2012) found that the bird component of wels catfish diet could be important for individuals specialising on pigeon predation. Our stable

Table 1. Numbers of nesting waterbird pairs of each species that could be potential prey of wels catfish in the Reserve, 2002–2019 and their local trends (based on at least three years of data). For comparison, the last column lists long-term trends in wintering numbers estimated by the International Wetland Census for the functional/ecological spatial wintering unit that includes the study area, plus contiguous spatial units (Zenatello et al. 2014). The vertical red line marks the period when wels catfish were first detected in the Reserve (2005–2008). Bold common names highlight species that were sampled for stable isotopes in our study, + indicates species present, but not counted.

Common name	Scientific	2002	2009	2016	2017	2018	2019	Local trend	IWC National
	name								Winter Trend
Mute swan	Cygnus olor	+	16	+	5	5	5	Decrease ($R^2 = 0.97$)	Increase
Great	Phalacrocorax	+	+	41	52	75	83	Increase $(R^2 = 0.97)$	Moderate increase
cormorant	carbo								
Red-crested	Netta rufina	+	+	+	7	6	8	Moderate increase ($R^2 = 0.25$)	Increase
pochard									
Eurasian coot	Fulica atra	20	9	+	7	10	10	Decrease ($R^2 = 0.63$)	Moderate increase
Common	Gallinula	+	+	+	50	50	50	Stable $(R^2 = 1)$	Moderate increase
moorhen	chloropus								
Mallard	Anas	20	+	+	21	20	20	Stable ($R^2 = 0.96$)	Increase
	platyrhynchos								
Great crested	Podiceps	35	+	+	22	12	13	Decrease ($R^2 = 0.88$)	Moderate increase
grebe	cristatus								
Little grebe	Tachybaptus ruficollis	+	+	+	6	7	8	Moderate increase ($R^2 = 1$)	Moderate increase
Water rail	<i>Rallus</i>	+	+	+	3	6	8	Moderate increase ($R^2 = 1$)	Increase
Purple heron	Ardea purpurea	6	+	+	8	12	9	Moderate increase ($R^2 = 0.56$)	Not wintering in Italy
Little bittern	Ixobrychus minutus	5	+	+	7	10	10	Moderate increase (R ² = 0.75)	Not wintering in Italy
Black-crowned night heron	Nycticorax nycticorax	50	17	10	15	27	19	Decrease ($R^2 = 0.53$)	Moderate increase

isotope data suggest that bird prey might not be limited to a few individuals with specialised predatory behaviour. However, both studies suffered from relatively low sample size of putative prey, which our analysis indicated could be downplaying the proportion of birds in catfish diet. Future studies should strive to increase putative prey sample size, aiming to get at least 15–25 stable isotope samples per prey category, but this might not yield a significant gain in the final estimate and may prove very challenging for some prey categories (e.g. birds). Sampling would also need to take into account the spatio-temporal variation in isotopic signals (Perga and Gerdeaux 2005) and the pulsed nature of prey sources (e.g. seasonal presence of breeding birds), by appropriately targeting sampling across space and time. Our simulations on sample size effects indicated that higher sample size would not dramatically decrease uncertainty around median estimates of diet proportions and that the bird component of the diet is likely to remain consistent with our findings. Additionally, our bioenergetic model yielded similar consumption rations as previously reported in literature. In any case, these two components make minor contributions overall to the uncertainty in bird consumption estimates. Uncertainties in the estimate of predator biomass remain the major source of error in the consumption estimate, but these might be a feature of field sampling and are unlikely to be reduced by increasing sampling effort, as this study already used a high number of sampling and removal events.

A more robust study setup, including further replicates of control and test areas, would be needed before drawing firm conclusions, but dietary proportions cannot be easily transposed from one area to another, so area-specific dietary studies would exponentially increase the fieldwork load. Adding replicates will also likely face a challenge in finding predator-free areas where the confounding effects of predator density could be excluded altogether. Wels catfish is currently widespread in Italian freshwaters, has heavily colonised the drainage of all main rivers in the country (Po, Arno, Tevere and Volturno Rivers) and is present in most of the protected areas of northern and central Italy. Where present, it tends to dominate the community of predator fishes (~ 30% of the whole fish community biomass, M. Milardi, unpublished data). Wels catfish and other introduced fish species are a major problem also for native fish diversity in Italian freshwaters (Milardi et al. 2018, 2019a, 2020a, 2020b, 2020c), but to date, little has been done to address this problem. A recent review by Cucherousset et al. (2018) underlined how the species is widespread and abundant also in the rest of its introduced range (e.g. France and Spain), where it was shown to have impacted on native fish, especially during migrations (Boulêtreau et al. 2020a, 2020b).

Local bird populations trends could be driven by population dynamics at a larger spatial scale (Milardi et al. 2019b). We tried to account for this uncertainty by considering the most recent assessment of long-term wintering population trends at the national scale (up to 2010), which highlighted how most waterbird species in our study area have trends decoupled from wider dynamics (Zenatello et al. 2014). This pattern was even more evident when examining the time series available for functional/ecological wintering units including our study area or in contiguous units, which all showed similar trends, suggesting that local-scale drivers, rather than larger-scale drivers, are driving local dynamics during the breeding season. Invasive freshwater predators have greater effects

than terrestrial ones because of native prey naïveté (Cox and Lima 2006) and the lack of experience with intense predation by introduced aquatic predators could partially explain why waterbirds are still nesting in our study site, despite the predation risk. Prey naïveté is less likely to apply to waterbirds given their mobility, as individuals may move to and from the native area of wels catfish, so predation avoidance strategies may be learned in or inherited from the native area (in a manner impossible for naive fish prey). However, both native and introduced aquatic predator strategies are likely to be similar and waterbirds might have limited capabilities to learn or deploy effective antipredator behaviour (e.g. because they are unable to detect large sit-and-wait predatory fish by sight or smell).

At present, it is still unclear whether all bird species could learn to avoid areas with high predation risk and, therefore, low reproductive success, as found for waterfowl exposed to northern pike predation (Dessborn et al. 2011). If most areas are heavily invaded, birds might have no choice but to try and breed where the catfish are, irrespective of their ability to gauge risks. If conservation areas are invaded, they might attract birds because of the perceived protection from human disturbance or suitable nesting habitat, yet ultimately, turn to sinks that lower waterbird species' reproductive success through predation by invasive fish. Other invasive fish species present in conservation areas can also have non-predatory effects on waterbirds through trophic cascades and habitat alteration (Maceda-Veiga et al. 2017; Milardi et al. 2020c). Despite their size, small reserves have potentially large conservation benefits (Volenec and Dobson 2020), but our results suggest that wels catfish might impact waterbird reproductive success through predation on bird chicks even in these areas. In our study area, wels catfish density did not decrease despite population control operations, which likely means that immigration of new individuals from Lake Iseo and growth and reproduction of individuals already in the Reserve, balanced the removal effort. However, it is likely that this effort has halted the invasion of wels catfish in the Reserve, mitigating the effects of predation on birds which were evident in the decline detected for some nesting species of waterbirds after the invasion onset. This suggests that post-invasion management is particularly challenging and highlights the value of non-permanent wetlands where periodic droughts offer the possibility to eliminate invasive fish and prevent recolonisation through mesh gates. Ultimately, our study suggests the need for an assessment of wels catfish impact on the conservation value of bird reserves and for more effective measures to mitigate this impact in its introduced range.

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Supplementary material I

Figures and Tables

Authors: Marco Milardi, Andy J. Green, Marco Mancini, Paolo Trotti, Mikko Kiljunen, Jyrki Torniainen, Giuseppe Castaldelli

Data type: Docx file.

- Explanation note: Figure S1. Daily average water temperature in the 'Torbiere del Sebino' Reserve in 2019. Figure S2. Wels catfish density trends detected through sampling events along the shoreline of different areas of the 'Torbiere del Sebino' Reserve in 2013–2019, using boat-mounted electrofishing. Each dot represents a single sampling event. Figure S3. Comparison between uninformative (right) and informative (left) priors. Informative priors were derived from our preliminary stomach content analysis and were tured into hyperparameters which were rescaled to have the same mean, but different variance, keeping the relative contributions the same. Figure S4. Sample size effects on the width of credible intervals and medians of posterior distributions for the 6 prey categories used in the mixing model. Putative prey sample size, expressed as values (boxplots, a) and percentage change in values (dot and line plots, b). Consumer sample size, expressed as percentage change in values (dot and line plots, c). Table S1. Length, weight, stable isotope ratios of C and N and percentage of each element and their ratio, for all the specimens sampled in this study. Table S2. Species-specific consumption, respiration and egestion/excretion parameters of the Wisconsin bioenergetic model for wels catfish used in our study. Table S3. Full model parameters for the Wisconsin bioenergetic model for wels catfish used in our study. Energy and water content of predator and prey items (Cummins and Wuycheck (1971), see Suppl. material 1: Table S3), dry weight to wet weight conversion factors (Hanson et al. 1997; Hartman and Brandt 1995), start and end weights for each cohort modelled, as well as cohort-specific dietary proportions derived from the stable isotope mixing models. Table 4. Average weight of adult males and females of each waterbird species nesting in the Reserve. Copyright notice: This dataset is made available under the Open Database License
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RESEARCH ARTICLE



How does familiarity in rhizobial interactions impact the performance of invasive and native legumes?

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Abstract

Mutualisms can be disrupted when non-native plants are introduced into novel environments, potentially impacting their establishment success. Introduced species can reassemble mutualisms by forming novel associations with resident biota or by maintaining familiar associations when they are co-introduced with their mutualists. Invasive Australian *Acacia* species in South Africa have formed nitrogen-fixing rhizobium mutualisms using both pathways.

Here we examined the contributions of novel vs familiar rhizobial associations to the performance of *Acacia saligna* across different soils within South Africa's Core Cape Subregion (CCR), and the concomitant impacts of exotic rhizobia on the endemic legume, *Psoralea pinnata*. We grew each legume with and without Australian *Bradyrhizobium* strains across various CCR soil types in a glasshouse. We identified root nodule rhizobium communities associating with seedlings grown in each treatment combination using next-generation sequencing (NGS) techniques.

Our results show that different CCR soils affected growth performances of seedlings for both species while the addition of Australian bradyrhizobia affected growth performances of *A. saligna*, but not *P. pinnata*. NGS data revealed that each legume associated mostly with their familiar rhizobial partners, regardless of soil conditions or inoculum treatment. *Acacia saligna* predominantly associated with Australian bradyrhizobia, even when grown in soils without inoculum, while *P. pinnata* largely associated with native South African *Mesorhizobium* strains.

Our study suggests that exotic Australian bradyrhizobia are already present and widespread in pristine CCR soils, and that mutualist limitation is not an impediment to further acacia invasion in the region. The ability of *P. pinnata* to sanction Australian *Bradyrhizobium* strains suggests that this species may be a good candidate for restoration efforts following the removal of acacias in CCR habitats.

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Keywords

Australian acacias, *Bradyrhizobium*, Cape Floristic Region, familiar associations, legume-rhizobium mutualisms, novel associations, *Psoralea pinnata*, woody invader

Introduction

Novel abiotic and biotic conditions can act as strong barriers to the successful establishment of introduced non-native species (Blackburn et al. 2011). Many plants rely on mutualisms to complete their life cycles, but these are often disrupted when they are introduced into new environments (Richardson et al. 2000; Parker 2001). The re-establishment/replacement of effective mutualisms in the new range depends on the availability and diversity of resident mutualists as well as the level of interaction specificity of both the introduced plant and resident native mutualists (Parker 2001; Le Roux et al. 2017a). When non-native plants have generalist requirements, they could easily form novel and effective associations with (usually generalist) resident mutualists. For example, in the Galápagos Islands, generalist invasive plants have successfully infiltrated native seed dispersal networks by attracting generalist native bird and reptile seed feeders (Heleno et al. 2013). Conversely, specialist non-native plants may only persist if their historical (i.e. native-range; or highly similar) associations are maintained (Rodríguez-Echeverría et al. 2011). This can happen when they are co-introduced with their native-range mutualists (i.e., so-called familiar associations; Le Roux et al. 2017a) or when they encounter resident mutualists that are phylogenetically closely-related to their native-range mutualists. For example, introductions of many pine trees in the family Pinaceae initially failed in Southern Hemisphere countries due to a lack of compatible ectomycorrhizal fungi (EMF) (Policelli et al. 2019). Upon introduction of pine-specific EMF, however, the trees established successfully and in many instances became widespread invaders (Richardson et al. 1994). In fact, recent work suggests that pines that are more invasive are also more reliant on EMF mutualists than less invasive pines (Moyano et al. 2020, 2021), thus highlighting the need for mutualism reassembly for invasion success.

The legume family (Fabaceae) comprises approximately 19,500 species, many of which form mutualistic associations with nitrogen-fixing soil bacteria, called rhizobia. Rhizobia form nodules on their hosts within which they fix atmospheric nitrogen, converting it into forms that their legume hosts can utilise in return for carbon-rich photosynthates. Legumes are also over-represented in alien floras, with approximately 1,189 naturalised species globally (9% of the 13,168 world's naturalised alien plants; Van Kleunen et al. 2015; Pyšek et al. 2017). Range expansion by non-native legumes is constrained by the availability of effective rhizobial symbionts (Simonsen et al. 2017; Lopez et al. 2020), with generalist legumes being more likely to become widespread than those with specialist requirements (Klock et al. 2015; Harrison et al. 2018; Wandrag et al. 2020). Highly invasive legumes, therefore, often form associations

with different rhizobia in their native compared to non-native ranges (e.g., Australian *Acacia* spp., *Cytisus* spp., *Leucaena* spp. and *Robinia* spp. in Brazil – de Faria and de Lima 1998; *Acacia pycnantha* in South Africa – Ndlovu et al. 2013; *Trifolium* spp. in New Zealand – Shelby et al. 2016). Conversely, specialist legumes usually fail to colonise new areas when they are not co-introduced with their co-evolved rhizobia (Parker 2001; Gehlot et al. 2013). The genus *Mimosa* provides a useful example. In India, non-native *Mimosa pudica* could not effectively associate with rhizobial strains associated with native Indian *Mimosa* species and only successfully established invasive populations following the introduction of its familiar rhizobial mutualist from South America (Gehlot et al. 2013; Melkonian et al. 2014).

Legume-rhizobium co-introductions appear to be commonplace. For instance, Australian acacias and their rhizobia have been co-introduced into South Africa (Ndlovu et al. 2013; Le Roux et al. 2016; Warrington et al. 2019), New Zealand (Weir et al. 2004; Warrington et al. 2019), Portugal (Rodríguez-Echeverría 2010; Crisóstomo et al. 2013), and into their non-native ranges in Australia (Birnbaum et al. 2016). In South Africa, Keet et al. (2017) found a high level of host selection for Australian bradyrhizobia by acacias, with widespread and localised *Acacia* species predominantly associating with one or two co-introduced Australian *Bradyrhizobium* strains. Acacias in South Africa are also known to form novel associations with the resident Core Cape Subregion (CCR) rhizobia (e.g., Ndlovu et al. 2013). Together, this suggests that acacias are promiscuous host plants capable of forming both familiar (i.e., with co-introduced rhizobia) and novel (i.e., with resident native rhizobia) associations in their new ranges.

South Africa's CCR is renowned for its exceptional plant diversity, attributed, in part, to a complex mosaic of soil conditions (Linder 2003, 2005; Cowling et al. 2009; Manning and Goldblatt 2012). The region is home to an estimated 764 native legumes, of which 83% are endemic (Manning and Goldblatt 2012), and is also recognised as a hub for exceptionally high endemic rhizobial diversity, with all major rhizobial genera found in the region (Kock 2004; Elliott et al. 2007; Gerding et al. 2012; Hassen et al. 2012; Kanu and Dakora 2012; Beukes et al. 2013; Lemaire et al. 2015, du Preez 2019). Heterogenous soil conditions in the CCR are also perceived as important in determining legume community diversity and composition (Dludlu et al. 2018b), and, in turn, that of native rhizobial communities through host-plant selection and soil abiotic conditions (Lemaire et al. 2015; Keet et al. 2017; Dludlu et al. 2018a). Bradyrhizobium strains are not common associates of native CCR legumes (Lemaire et al. 2015). However, bradyrhizobia tend to have cosmopolitan distributions within soils (e.g., Le Roux et al. 2021) due to their low sensitivity to fluctuations in soil characteristics, such as pH, which, in turn, may benefit Bradyrhizobium specialists like introduced Australian acacias (Lange 1961; Lafay and Burdon 2001; Birnbaum et al. 2016; Le Roux et al. 2016; Keet et al. 2017; Kamutando et al. 2019). This low edaphic sensitivity, together with the presence of a compatible host, may therefore facilitate both the survival of exotic Bradyrhizobium strains and, subsequently, the successful colonisation by introduced acacias. Indeed, acacia invasions often result in localised enrichment of Bradyrhizobium strains in the CCR (Slabbert et al. 2014; Keet et al. 2017; Le Roux et al. 2018) which can lead to

homogenisation of rhizobial communities and lower native rhizobial diversity (Le Roux et al. 2018; Kamutando et al. 2019). This may facilitate the successful colonisation of other *Acacia* species as acacias often utilise the same bradyrhizobia interchangeably (Keet et al. 2017; Warrington et al. 2019) while negatively impacting native legumes, particularly when they are not compatible with *Bradyrhizobium* (e.g., Rodríguez-Echeverría et al. 2012). Decreased native rhizobial diversity and a concomitant increase in bradyrhizobia may also hamper the ability of some native legumes to sanction ineffective strains (Denison 2000; Westhoek et al. 2021).

Despite the wealth of information on acacias and their rhizobia in the CCR, it remains unclear how the presence of Australian rhizobia affects the growth performance of invasive acacias and co-occurring native CCR legumes. Here, we aimed to address this knowledge gap. A glasshouse experiment was set up to compare the performance of invasive *Acacia saligna* and native *Psoralea pinnata* grown in different uninvaded CCR soil types, with or without the presence of Australian *Bradyrhizobium* strains. Next generation sequencing (NGS) approaches were used to characterise the root nodule communities of both legumes under these different growth conditions. We hypothesised that the performance of *A. saligna* would be enhanced when forming familiar associations under treatments that received Australian bradyrhizobia inoculum while the performance of *P. pinnata* would be negatively impacted by the presence of exotic mutualists.

Methods

Study system

Acacia saligna (Labill.) Wendl., commonly known as Port Jackson willow, is native to south-western Australia and is invasive in many of the world's Mediterranean regions. Of the 15 invasive Australian acacias present in South Africa, *A. saligna* has the fifth largest distribution (Richardson et al. 2015). The species forms dense thickets with many devastating impacts on above- and belowground biodiversity and edaphic characteristics (Le Maitre et al. 2011). *Acacia saligna* is promiscuous and associates with a wide range of rhizobia, but, like most Australian acacias, is commonly nodulated by *Bradyrhizobium* strains (Marsudi et al. 1999; Lafay and Burdon 2001; Keet et al. 2017; Stępkowski et al. 2018).

Psoralea pinnata L., commonly known as fountain bush, is native to the southwestern CCR and is found in a variety of fynbos vegetation types, particularly on acidic, nutrient-poor, sandstone-derived soils, or on richer shale soils (Bello et al. 2017). The species is predominantly nodulated by *Mesorhizobium* strains (Kanu and Dakora 2012; Lemaire et al. 2015), however, associations with *Paraburkholderia* (previously *Burkholderia*) and *Rhizobium* strains have also been documented (Kanu and Dakora 2012). Interestingly, *P. pinnata* has been introduced to western and eastern Australia where it has become naturalised, and has been identified as a potential invader, including in habitats where *A. saligna* naturally occurs (Stirton et al. 2015). No information is currently available on the identity of rhizobia nodulating *P. pinnata* in Australia. In the CCR, it is frequently found growing in sympatry with Australian acacias (Staci Warrington, personal observation). Differences in the rhizobial associations of these two legumes, together with their sympatric distributions in Australia and South Africa, make them interesting systems to study the impact of familiar and novel mutualist associations on the performance of native and invasive species.

Soil collection

We collected soils from four pristine CCR areas to capture a range of abiotic conditions. As a fifth soil type, we also sampled soils directly beneath *P. pinnata* plants to capture the potential abiotic and biotic conditions induced by this species. These soils were collected during October 2018 across the Stellenbosch Winelands and Overberg districts of the CCR (see Suppl. material 1: Fig. S1 for site map and Suppl. material 1: Table S1 for site details).

The four non-*Psoralea*-conditioned soil types were collected at sites where neither *P. pinnata* nor *A. saligna* were present (other native legume species were observed at these sites). These sites were in the Grootbos Private Nature Reserve (sandy soils), Kogelberg Nature Reserve (sandy/loamy soils), Rustenberg Winery (clay soils), and Vergelegen Wine Farm (loamy soils). Within each site, soils were collected from four sampling points that were approximately 5m apart. The topsoil (the top 5cm of soil) was scooped aside and 25L of soil excavated at each sampling point. These were then mixed for each site and stored within a sterile storage container (i.e., 100L of soil in a single container per site). All soil sampling equipment was rinsed and sterilised with 70% ethanol between collections.

'Psoralea-conditioned soils' were collected directly beneath five different *P. pinnata* individuals spread across three different sites: Prawn Lake in Hermanus, Kogelberg Nature Reserve, and Vergelegen Wine Farm (Suppl. material 1: Fig S1, Table S1). Individual plants within each site were a minimum of 50 m apart, were over 1.5 m tall, and were part of a well-established population. The excavation procedure was the same as for the other four soil types. Twenty litres of soil were collected from within a 1m radius of each of the five shrubs, bulked and mixed thoroughly to make up 100L of soil per site in total.

All soils were separately sieved through a 4 mm mesh to remove any plant debris and rocks. All equipment were sterilised with 70% ethanol between sieving of individual soils. Soils were then returned to storage containers and stored at room temperature for a period of three months before commencing the glasshouse experiment.

Glasshouse experimental setup

We placed a layer of standard unsterilised store-bought drainage chips, followed by two litres of site-specific soil, into plastic gardening pots (18 cm diameter \times 15.5 cm height), which were each placed onto a water-collecting saucer. This was done for a

total of 40 pots per soil type (five soil types; total n = 200). We chose to use whole soils (instead of soil inocula) to maintain all soil abiotic conditions that may favour native rhizobia (i.e., to which they are adapted), and to simulate the novel conditions under which co-introduced rhizobia would need to operate. All equipment used during this process was sterilised with 70% ethanol between potting of the different soil types. All pots were then watered with tap water until soils were saturated.

Seeds of *A. saligna*, collected from invasive CCR populations, were obtained from the Agricultural Research Council's Plant Protection Research Institute (ARC-PPRI) in Stellenbosch. *Psoralea pinnata* seeds, collected from populations across the Cape Peninsula in the CCR, were supplied by Silverhill Seeds in Kenilworth, Cape Town. Prior to planting, all seeds were surface-sterilised (Birnbaum et al. 2012), and scarified (*P. pinnata* – Siva et al. 2014; *A. saligna* – Rincón-Rosales et al. 2003). We planted four seeds of each species into 20 individual pots/soil type. Five weeks later we randomly removed all but one seedling if multiple seeds had germinated in each pot. In a few pots, no seeds germinated. To make up for these losses, extra seedlings removed from pots with high germination rates were transplanted into these pots, within the same species × site × inoculum treatment combinations (see Suppl. material 1: Table S2 for further details).

To ensure that rhizobial communities were still present in soils post-storage, we collected fresh soil from each site and added these to the pots as a soil inoculum (van de Voorde et al. 2012). Soil collections and sieving in the field were done as described above, except that only 60L of each soil type was collected. We added 0.2L of this fresh soil to the relevant pots (i.e., each pot containing a specific soil type received soil inoculum of the same soil type) for both species. This was done six weeks post-sowing once all seeds had germinated (Klock et al. 2015; Le Roux et al. 2018) and to ensure seedlings were tall enough to avoid being smothered by the added soil. We sterilised all equipment with 70% ethanol between additions of different soil inocula.

Australian Bradyrhizobium inoculum preparation

An Australian inoculum cocktail, consisting of five *Bradyrhizobium* strains that we previously isolated from *Acacia dealbata*, *A. decurrens*, and *A. melanoxylon* in Australia (Warrington et al. 2019; see Suppl. material 1: Table S3 for more details), was applied to the seedlings. These isolates are from the so-called Clade I *Bradyrhizobium* (*sensu* Stępkowski et al. 2018), an endemic lineage (Mishler et al. 2014) that houses the primary mutualists of many Australian *Acacia* species (Stępkowski et al. 2018; Le Roux et al. 2021). Therefore, although the strains we used were not isolated from *A. saligna*, they likely represent bacteria that are highly compatible with this legume. Indeed, many Australian acacias appear to share the same Clade I *Bradyrhizobium* strains interchangeably and with similar efficacy (Wandrag et al. 2013; Keet et al. 2017; Warrington et al. 2019). We grew these strains in separate Yeast Mannitol liquid broths in a shaking incubator (155 rpm) at 28 °C for a period of 5 days. We mixed 15mL of each strain, creating a rhizobial cocktail (75 mL) which was diluted in 1,425mL dH₂O to make up 1.5L of inoculum. Using a pipette, we added 5mL of this

inoculum to 10 of the 20 pots per species per soil type (n = 10 for each species × soil type × inoculum addition treatment combination). The remaining 10 pots for each soil type received 5mL sterile Yeast Mannitol broth that had been diluted in the same manner as the inoculum. Australian inoculum was first added seven weeks post-sowing as this allowed sufficient time (six weeks) for all seedlings to germinate (Klock et al. 2015; Le Roux et al. 2018) and one week for the bacterial communities added through the soil inoculum to establish. Inoculum addition was repeated four weeks later.

Glasshouse experiment protocols and measurements

All pots were randomly placed in a glasshouse exposed to ambient light and temperature conditions, and we randomised all pots weekly to minimise microclimate effects on seedling growth. Prior to soil inoculum addition, all pots were watered *ad libitum* two to three times a week with tap water. After adding the soil inoculum, a stringent watering system was put in place whereby we individually watered each pot every two days to minimise cross-contamination. All pots received the same amount of water. Randomisation took place prior to watering when saucers were dry to further minimise cross-contamination through spillage.

Plants were grown for a total of 17 weeks. Prior to harvesting plant material, we measured seedling height (defined as the length between the point where the stem exits the soil surface and the furthest apical meristem along the main stem). During seedling harvest we made every effort to minimise nodule loss and damage to seedling root systems. Each pot was gently tapped to loosen the soils from the sides of the pot. The seedling and the soil were then easily removed from the pot and placed onto a clean surface. Here, soils surrounding the root system were loosened further until they could gently be shaken from the roots. Any roots that had broken off during this process were collected. These roots, and those still attached to the plant, were rinsed in water to remove any remaining soil and tapped dry with tissue paper. Root nodules for each seedling were counted, removed, and placed into tubes containing silica gel for desiccation. Finally, we divided seedling biomass into root and shoot fractions and placed these into separate brown paper bags, followed by drying in an oven at 55 °C for one week. Dried shoot and root (excluding nodules) material, and desiccated root nodules were weighed separately. Altogether, the growth performance measurements included seedling height, seedling shoot dry biomass, seedling root dry biomass, seedling total dry biomass, and root:shoot ratios.

As a proxy for biological nitrogen fixation (BNF), we analysed $\delta^{15}N$ isotopic signatures (Lötter et al. 2014). Dried phyllode (*A. saligna*) and leaf (*P. pinnata*) material were removed after weighing of shoot material. This material was processed and sent to iThemba Labs (Pretoria, South Africa) for isotopic analysis (see Suppl. material 1: ESM1 for details). Generally, $\delta^{15}N$ values are expressed as parts per thousand deviation from the ¹⁵N composition of atmospheric nitrogen (defined as 0‰; Mariotti 1983). The lighter isotope (¹⁴N) is preferentially incorporated by nitrogenase during N-fixation. Consequently, $\delta^{15}N$ minima (i.e., extreme negative values) are reached when plant-incorporated N is derived solely via BNF (i.e., atmospheric N), and values increase with increasing contribution of soil-derived N (Unkovich 2013). δ^{15} N values close to zero or negative are indicative of BNF while positive values suggest that NH₃ was predominantly assimilated from soil nutrient pools. These δ^{15} N measurements, together with the nodule count and nodule total dry biomass measurements, were used as proxies to estimate differences in nitrogen assimilation through BNF within specific soils in the presence and absence of Australian bradyrhizobia.

Statistical analyses of growth performance and BNF measurements

All statistical analyses were conducted in the R statistical environment (v3.4.4; R Core Team 2021) and separately for each legume species.

To investigate the effect of Australian inoculum and soil type on the overall growth performance (i.e., seedling height, seedling shoot dry biomass, seedling root dry biomass, seedling total dry biomass, root:shoot ratios), and BNF (i.e., number of nodules, nodule total dry biomass, δ^{15} N) of the seedlings, we ran models using Australian inoculum (addition or no addition), soil type (Grootbos, Kogelberg, Rustenberg, Vergelegen, and Psoralea-conditioned), and their interaction as main effects. Factorial ANOVAs followed by Tukey HSD post-hoc tests were used for most of the performance and proxies of BNF measurements for both species, except for seedling total dry biomass for A. saligna seedlings, root:shoot ratio and nodule number for P. pinnata seedlings, and seedling root dry biomass for both species. Generalised linear models with a Gamma family data distribution (link = inverse) were used for seedling root dry biomass, seedling total dry biomass and root:shoot, and a generalised linear model with a negative binomial distribution for nodule number (See Suppl. material 1: Table S4 for details). The negative binomial data distribution family was chosen for nodule number to account for over-dispersion of data (Rodriguez 2013). We determined the overall effect size of Australian inoculum addition and soil type, and their significance, for all performance measures using the Anova function (type II sum of squares) in the car R package (Fox and Weisberg 2018). Finally, pairwise contrasts between levels of the main effects were determined using the emmeans function in the emmeans R package (Lenth et al. 2018).

To determine the relative contribution of the number of nodules to seedling growth performance and BNF under the two inoculum treatments; that is, the average gain in performance with increased nodulation (i.e., rhizobial efficacy), we regressed each growth and BNF measurement against nodule number (continuous predictor) and Australian inoculum addition treatment (categorical predictor) using generalised linear models (See Suppl. material 1: Table S4 for details on the data distribution families used for each measurement). Data from all soil types were combined for these analyses. We determined overall effect sizes and the significance of each main effect using the *Anova* function (type II sum of squares) for each performance and BNF measurement for each species, except for *A. saligna* seedling shoot dry biomass, seedling root dry biomass, seedling total dry biomass, root:shoot and nodule total dry biomass, for which type III sum of squares ANOVAs were used due to the significant interaction term.

DNA extraction and next-generation sequencing (NGS) of root nodule and inoculum rhizobia

To determine the identity and abundance of rhizobial strains within root nodules of *A. saligna* and *P. pinnata*, we pooled between 3–5 nodules from each seedling within a particular species × soil × inoculum treatment combination for each of the 20 combinations (i.e., 20 samples in total, each comprising 30–50 nodules). For DNA extraction, desiccated nodules were tissuelysed into a fine powder to create a homogenous mixture of nodule material. We extracted DNA from these mixtures using the DNeasy Plant Mini Extraction Kit (Qiagen, supplied by White Head Scientific, Cape Town, South Africa) according to manufacturer specifications.

To extract DNA of the Australian *Bradyrhizobium* isolates used in the inoculum, we grew all five strains from glycerol stocks in separate Yeast Mannitol broths in a shaking incubator (155 rpm) at 28°C until there was sufficient bacterial growth (indicated by a milky, turbid colour change). We extracted DNA from these cultures using the Sigma Gen-Elute Bacterial Genomic DNA kit (Sigma-Aldrich Co. LLC, USA), according to manufacturer specifications. Isolated DNA concentrations and quality were checked using a NanoDrop ND-1,000 UV-Vis Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). These samples were used as 'reference' strains in subsequent analyses.

We amplified the nodulation C (*nodC*) gene for NGS, using the primers nod-CF12F (5'-CCG GAT AGG MTG GKB CCR TA-3') and nodCRI2R (5'-GTG CAC AAS GCR TAD RCC TTC AH-3'), with sample-specific barcodes in the forward primer. This gene has been successfully utilised for taxonomic identification of rhizobia in both the *alpha*- and *beta*-Proteobacteria (Le Roux et al. 2017b). Amplification and sequencing were performed at the Molecular Research LP next-generation sequencing service (www.mrdnalab.com, Shallowater, TX, USA) on an Illumina MiSeq instrument following manufacturer protocols. PCR conditions and sequencing protocols can be found in Suppl. material 1: ESM2.

NGS bioinformatics

NodC sequences were joined, and sequences < 150bp in length or with ambiguous base calls were removed. Sequences were quality filtered using a maximum expected error threshold of 1.0 and dereplicated. The dereplicated or unique sequences were denoised; unique sequences identified with sequencing or PCR point errors were removed; and chimeras removed, yielding zero-radius Operational Taxonomic Units (zOTUs).

Since no reference database exists for *nodC* sequences, each zOTU was blasted against the NCBI's GenBank database (http://blast.ncbi.nlm.nih.gov/Blast) to determine its potential taxonomic identity. All non-nitrogen-fixing bacteria were removed from the dataset so that only rhizobia were considered in subsequent analyses. We clustered the remaining zOTUs at 97% DNA sequence similarity via the nearest-neighbour algorithm, based on pairwise sequence similarity distances calculated with the Needleman-Wunsch algorithm in mothur v1.44.1 (Schloss et al. 2009).

We found many low-abundance OTUs (<100 sequence reads/sample). Therefore, the relative abundance of each OTU within individual samples (i.e., each species × soil × inoculum treatment combination) was calculated and all rare OTUs, that is, those making up less than 5% of the cumulative abundance per sample for all samples, were removed from the dataset. This resulted in a final dataset comprising ten OTUs that occurred at a relative abundance of > 5% in at least one sequenced sample.

Phylogenetic analysis

Blast results indicated that most of our ten OTUs belonged to the genus *Bradyrhizobium*. In order to determine the possible geographic origin of the these strains, we obtained additional *nodC* sequence data previously generated using the same approaches outlined above (i.e., the same primers and NGS platform) of *Bradyrhizobium* strains isolated from the root nodules of invasive acacias (Keet et al. 2017) and acacia-invaded soils in South Africa (Le Roux et al. 2018), as well as from root nodules of the *Acacia* species (from which some of our inoculum strains were isolated) in Australia (*A. decurrens* and *A. melanoxylon*; Urbina and Klock, unpublished). We downloaded *nodC* sequence data from GenBank for *Bradyrhizobium* strains previously isolated from native CCR legumes (Lemaire and Muasya, unpublished). We also included one *Mesorhizobium nodC* sequence as an outgroup. These additional sequence data were trimmed and aligned with our data using Clustal W in BioEdit (Hall 1999).

The best-fit nucleotide substitution model for the aligned dataset was determined using JModelTest (Posada 2008) and Akaike information criterion (Akaike 1973). The HKY + G + I (Hasegawa et al. 1985) model was identified as the best fit model. We then used MEGA X (Kumar et al. 2018; Stecher et al. 2020) to reconstruct a phylogeny using this model and maximum likelihood search criteria. Bootstrap values were calculated using the majority rule consensus method to assess topological support of the phylogeny.

OTU comparisons between treatments

To investigate the prevalence of the Australian inoculum *Bradyrhizobium* strains in association with *A. saligna* seedlings, the relative abundances of dominant inoculum OTUs were compared between inoculum treatments. These comparisons were only done for SW OTU1 and SW OTU6 as these were the only OTUs present within the reference samples with a relative abundance > 5% (see Results). We combined the relative abundance data for all soils and compared these between the two inoculum treatments using a paired t-test and a Wilcoxon signed-rank test for SW OTU1 and SW OTU6, respectively.

The relative abundances of each of the ten individual OTUs were compared between the different species × Australian inoculum addition treatment combinations for all soil types combined. This was done to determine whether *A. saligna* and *P. pinnata* differed in their rhizobial associations and whether these associations differed in the presence of the exotic *Bradyrhizobium* (i.e., inoculum addition). We performed these comparisons using a permutational multivariate analysis of variance (PERMANOVA) in the *vegan* R package (Oksanen et al. 2013). A distance matrix for relative abundance data of all ten OTUs was developed following the Bray-Curtis dissimilarity method using the *vegdist* function and used this matrix as the response variable in the PERMANOVA with Australian inoculum addition treatment (inoculum added or not added) and host species (*A. saligna* and *P. pinnata*), as well as their interaction, as main effects. The PERMANOVA was run using the *adonis2* function with 999 permutations. We performed post-hoc analyses using the *simper* function to elucidate which OTUs were contributing most to any dissimilarities in the nodule rhizobial community composition. All functions form part of the *vegan* R package.

Results

Australian inoculum addition and soil type

As growth performance measurements were frequently significantly correlated for both species (results not shown), we only report on seedling total dry biomass (significantly correlated with seedling root and shoot biomasses and seedling height) and root:shoot ratios. Similarly, only nodule number (which correlated with nodule total dry biomass) and δ^{15} N are reported as proxies of BNF (also see Suppl. material 1: Tables S5, S6; Figs S3, S4). Increases in seedling total dry biomass accompanied by low root:shoot ratios are interpreted as advantageous as this indicates that plants invested more heavily into shoot biomass than root biomass. This is often due to a higher nutrient availability either through increased soil nutrient availability or through effective rhizobial associations (Friel and Friesen 2019).

There was a significant inoculation effect leading to increased nodule formation in *A. saligna* seedlings ($F_{(1)} = 5.638$, p = 0.0201; Suppl. material 1: Table S5). However, this did not translate into differences between inoculation treatments within each soil type (Fig. 1) and there was a significant interaction between inoculation and soil type for *A. saligna* δ^{15} N values ($F_{(1)} = 2.507$, p = 0.0488; Fig. 1; Suppl. material 1: Table S5). Counterintuitively, this was primarily driven by an increase in δ^{15} N values in *Psoralea*-conditioned soils for those seedlings that received Australian inoculum (Fig. 1). In contrast to *A. saligna*, there was never a significant Australian inoculum addition effect nor a significant interaction between inoculum addition and soil type for any *P. pinnata* growth performance and δ^{15} N measurements.

Soil type significantly influenced all growth performance and δ^{15} N measurements of both species (Suppl. material 1: Table S5). Both species appeared to have significantly higher total biomass and nodule numbers, and lower δ^{15} N values when grown in soils from Rustenberg and *Psoralea*-conditioned soils (Fig. 1). Root:shoot ratio responses were largely similar across all five soil types for both species, with differences in biomass allocation only manifesting between different inoculum treatments of the same (*A. saligna* in *Psoralea*-conditioned soils) or different (*P. pinnata* in Grootbos, Rustenberg, and Vergelegen) soils (Fig. 1).



Figure 1. Comparisons of seedling performances between Australian inoculum addition and soil type treatment combinations. Seedling total dry biomass (**A**, **B**), root:shoot ratio (**C**, **D**), number of nodules (**E**, **F**) and δ^{15} N (**G**, **H**) measurements for *Acacia saligna* (left) and *Psoralea pinnata* (right) for each site (Grootbos, Kogelberg, Rustenberg, Vergelegen and *Psoralea*-conditioned (Pc) soils) by inoculum treatment (red – Australian inoculum added; blue – no inoculum added) combination.

Rhizobia efficacy

For *A. saligna*, nodule number was a significant predictor of seedling total dry biomass ($\chi^2_{(1)} = 43.862$; p < 0.0001) and root:shoot ratios ($\chi^2_{(1)} = 14.8465$; p = 0.0001), both of which increased with increasing nodulation, and $\delta^{15}N$ values ($\chi^2_{(1)} = 4.2034$; p = 0.0403), which decreased with increasing nodulation (Fig. 2; Suppl. material 1: Table S6). While Australian inoculum addition on its own was not significant for



Figure 2. Comparisons of rhizobial efficacy between inoculum addition treatments. The contribution of nodules to seedling total dry biomass (**A**, **B**), root:shoot ratios (**C**, **D**) and δ^{15} N (**E**, **F**) of *Acacia saligna* (left) and *Psoralea pinnata* (right) for all sites combined and the influence of inoculum treatment (red – Australian inoculum added; blue – no inoculum added) on each. P-values for interaction terms (i.e., nodule number*inoculum) are provided.

any of the measurements, there were significant interactions between the number of nodules and Australian inoculum addition for *A. saligna* seedling total dry biomass ($\chi^2_{(1)} = 11.692$; p = 0.0006) and root:shoot ($\chi^2_{(1)} = 4.7948$; p = 0.0285), but not for $\delta^{15}N$ ($\chi^2_{(1)} = 0.2406$; p = 0.6237). *Acacia saligna* seedlings that did not receive inoculum gained more total biomass and root:shoot ratios than those seedlings that did receive inoculum. That is, for a given number of nodules formed, these values were higher for uninoculated seedlings than for inoculated seedlings, as indicated by the difference in slope for these two treatments (Fig. 2; Suppl. material 1: Table S6, Fig. S4). This increase in total biomass for uninoculated *A. saligna* seedlings is likely driven by

an overall higher investment in belowground rather than aboveground growth for all soil types, as shown by the root:shoot ratios of uninoculated seedlings tending to be higher than inoculated seedlings (Fig. 1), though these were non-significant.

For *P. pinnata* seedlings, inoculation as a main effect, as well as the interaction between the number of nodules formed and Australian inoculum addition, were non-significant for both measures of seedling growth performance and the $\delta^{15}N$ values (Suppl. material 1: Table S6). Only nodule number as a main effect was significant for all measurements (p < 0.0001 in all instances; Suppl. material 1: Table S6).

NGS Bioinformatics and phylogeny

After data quality-checking, the *nodC* dataset generated 272 zOTUs. Removing zO-TUs representing non-nitrogen-fixing bacteria (34.6% of zOTUs), followed by clustering the remaining zOTUs at 97% DNA similarity level, and the removal of singleton/doubleton OTUs (leaving a total of 45 clustered OTUs) and OTUs with < 5% relative abundance per sample (77.8% of clustered OTUs) for all samples, resulted in 943,739 sequences representing ten OTUs.

Blast results for these OTUs indicated that they belonged to the genera *Bradyrhizobium* (five OTUs), *Mesorhizobium* (four OTUs), and *Rhizobium* (one OTU) (Suppl. material 1: Table S7). Of these, only two OTUs (SW OTU1 and SW OTU6) were present in the reference samples used in the Australian inoculum with a relative abundance > 5%. These blasted to *Bradyrhizobium* sp. CPI240 and *Bradyrhizobium* sp. CPI241, respectively, previously isolated from *Acacia* species in Australia (Barrett et al. 2016). SW OTU1 and SW OTU2 were the dominant strains isolated from nodules of *A. saligna* and *P. pinnata*, respectively, with blast results identifying SW OTU2 as being closely related to *Mesorhizobium* sp. 969n9 previously isolated from South African legumes (Lemaire & Muasya, unpublished) (Suppl. material 1: Table S7). Blast results also revealed that *A. saligna* and *P. pinnata* associated with native CCR *Mesorhizobium* strains (SW OTU17) in Grootbos soils, and Australian *Bradyrhizobium* strains (SW OTU1) in Vergelegen soils, respectively. These are the only instances of novel associations identified in this study (Fig. 3).

The *Bradyrhizobium nodC* phylogeny yielded many unsupported nodes, likely because of the short length (312 bp) of the NGS reads (Fig. 4). However, it provided high support for two distinct clades, one including *Bradyrhizobium* strains previously isolated from native CCR legumes and the other including *Bradyrhizobium* from this study and strains previously isolated from acacia-invaded soils (JLR OTUs in Fig. 4; Le Roux et al. 2018) and acacia -associated root nodules in South Africa (JHK OTUs in Fig. 4; Keet et al. 2017), and acacia root nodules in Australia (HU_MG accessions in Fig. 4; Urbina and Klock, unpublished). Several of our OTUs (i.e., SW OTUs) clustered with these previously reported acacia OTUs with high support. Specifically, the dominant *Bradyrhizobium* OTU found in this study, SW OTU1, clustered with the dominant OTUs identified by Keet et al. (2017), Le Roux et al. (2018) and Urbina and Klock (unpublished). The second most abundant *Bradyrhizobium* OTU in our study, SW OTU6, clustered with an abundant OTU identified by Keet et al. (2017) and Urbina and Klock (unpublished) (Fig. 4).



Figure 3. Relative abundances of the dominant OTUs associating with *Acacia saligna* and *Psoralea pinnata* seedlings. Heatmap based on the relative abundances of the ten rhizobial OTUs identified in this paper. Darker shades represent higher relative abundances. OTUs are arranged according to country of origin (top x-axis) based on blast results. Y-axis labels show the reference samples used as inoculum as well as the 20 species × soil × inoculum addition treatment combinations. OTU labels and genus identity based on blast results are given on the bottom x-axis.

Root nodule rhizobial composition comparisons

The relative abundances of the two dominant OTUs, SW OTU1 (259,830 sequence reads) and SW OTU6 (10,540 sequence reads), found in the reference samples, did not differ in *A. saligna* root nodules between the two inoculum treatments (SW OTU1: Paired t-test, $t_{(5)} = 1.034$, p = ns; SW OTU6: Wilcoxon signed-rank test, W = 11; p = ns).

PERMANOVA indicated that Australian inoculum addition did not significantly change the relative composition of nodule OTU communities ($F_{(1,16)} = 0.405$; p = ns). However, the composition of nodule OTU communities differed significantly between host plant species ($F_{(1,16)} = 21.485$, p < 0.001) (Suppl. material 1: Table S8). Post-hoc analysis using the *simper* function showed that this significant host species effect was largely driven by SW OTU1 and SW OTU2 which accounted for 35.35%



Figure 4. Phylogenetic tree showing relationships between this study's *Bradyrhizobium* strains and those isolated by similar local/international research. Maximum Likelihood phylogenetic tree showing the relationships between *nodC* sequences of *Bradyrhizobium* strains for this study (SW OTU strains) as well as those sequences previously isolated from acacia soils (JLR OTU strains), acacia nodules (South Africa: JHK OTU strains; Australia: HU_MG accessions) and CCR legumes ('BL' accessions) as indicated by the shaded blocks in the corresponding table. Tree is drawn to scale with branch length measured in the number of substitutions per site. Nodal support is given as bootstrap values.
and 34.01%, respectively, of the total compositional dissimilarity in nodule rhizobial communities between the two legume species (Suppl. material 1: Table S9). Specifically, *A. saligna* associated predominantly with *Bradyrhizobium* SW OTU1, while *P. pinnata* predominantly associated with *Mesorhizobium* SW OTU2. The remaining OTUs each accounted for less than 10% of the dissimilarity of root nodule communities between the two legume species (Fig. 3; Suppl. material 1: Table S9).

Discussion

Australian acacias have been co-introduced with their Bradyrhizobium strains into several regions across the globe (Rodríguez-Echeverría 2010; Crisóstomo et al. 2013; Warrington et al. 2019), and these exotic bradyrhizobia have the potential to negatively impact native legumes by outcompeting native rhizobia or by forming ineffective novel associations with them (Rodríguez-Echeverría et al. 2012; Le Roux et al. 2017a). In this study, however, we reject our hypothesis that the presence of exotic Australian bradyrhizobia negatively impacts the native CCR legume, Psoralea pinnata, as this species is successful at sanctioning these bacteria in favour of its familiar native Mesorhizobium strains. Whether the opposite is true for invasive Australian Acacia saligna (i.e., increased performance due to familiar associations) cannot be wholly resolved here as it formed familiar associations with Australian bradyrhizobia in all soils, with only one instance of a novel association. This suggests that the limited effects of inoculum addition on performance is likely due to the known widespread presence of exotic Australian bradyrhizobia in acacia-invaded CCR soils (Ndlovu et al. 2013; Keet et al. 2017; Le Roux et al. 2018; Warrington et al. 2019). Our study suggests that these exotic bradyrhizobia are also present in pristine, uninvaded, CCR soils.

Recent evidence suggests that nodule communities are largely made up of so-called core microbiomes, consisting of the most compatible and effective symbionts of the host (Shade and Handelsman 2012; Rodríguez-Valdecantos et al. 2017), likely as a result of host selection coupled with neutral processes such as ecological drift (Ramoneda et al. 2020). Although these mechanisms were not explicitly tested here, our NGS results and OTU comparisons suggest that Bradyrhizobium and Mesorhizobium are the core symbionts (through host selection) of A. saligna and P. pinnata, respectively (for A. saligna also see: Marsudi et al. 1999; Lafay and Burdon 2001; Keet et al. 2017; and for *P. pinnata* also see: Kanu and Dakora 2012; Lemaire et al. 2015). It is likely that the predominant association of *P. pinnata* with *Mesorhizobium*, and its ability to successfully sanction exotic Bradyrhizobium associations, resulted in the negligible effect of Australian bradyrhizobia on its performance. Although the exact mechanisms are not known in this case, legumes are known to minimise the impact of ineffective rhizobial associations through partner selection (Heath and Tiffin 2009; Sachs et al. 2010) and sanctioning (Kiers et al. 2003; Sachs and Simms 2006). Additionally, our results show that CCR Mesorhizobium strains can co-exist with Australian bradyrhizobia. Both

Mesorhizobium and *Bradyrhizobium* have adaptations to seasonally dry, acidic soils, likely resulting in overlapping distributions (Rodríguez-Echeverría et al. 2003; Dludlu et al. 2018a). In contrast, other native CCR legumes have been found to be unable to limit associations with novel, and potentially less effective, Australian *Bradyrhizobium* strains in invaded sites (Le Roux et al. 2016). *Psoralea pinnata* is often one of the few native CCR legumes to regenerate through passive restoration in sites where Australian acacias have been cleared (Reinecke et al. 2008), highlighting its ability to survive in *Bradyrhizobium*-enriched soils. Therefore, the impact of co-invading acacias and bradyrhizobia is likely to be negligible on this native legume, at least from a nitrogenfixing symbiosis perspective, making it a good candidate for active restoration.

Aside from the prevalence of familiar rhizobial associations, both A. saligna and P. pinnata formed a single novel association within Grootbos and Vergelegen soils, respectively. When grown in Grootbos soils, A. saligna plants had nodules containing high relative abundances of Mesorhizobium SW OTU17 regardless of inoculum treatment (Fig. 4; Suppl. material 1: Table S10), one of only two non-Bradyrhizobium associations identified for this species in this study. In Vergelegen soils, when Australian inoculum was not added, P. pinnata associated with the dominant Mesorhizobium SW OTU2. However, associations predominantly involved the Bradyrhizobium SW OTU1 when Australian inoculum was added. Nonetheless, both legume species had similarly poor growth performances under both Grootbos and Vergelegen soils, even when forming familiar associations. Therefore, while it is intuitive to attribute the poor growth performances to these novel rhizobial associations, which appear to be potentially bordering on parasitism (Denison and Kiers 2004; Rodríguez-Echeverría et al. 2012), the similarly poor seedling performances in these soils, even when forming familiar associations, suggests that site-specific edaphic conditions may have played a greater role.

Overall, differences in soils, rather than inoculum addition, largely explained differences in the growth performance and BNF proxies (i.e., δ^{15} N values and nodule numbers) of both legume species (Fig 1). Both species generally performed poorly in Grootbos, Kogelberg and Vergelegen soils and performed best in Rustenberg and Psoralea-conditioned soils, regardless of inoculum addition or associations with preferred rhizobia (Fig. 1, 3). Our rhizobial efficacy results (Fig. 2) showed that δ^{15} N values of both species decreased with increased nodulation, suggesting BNF is occurring, which is also suggested by near zero δ^{15} N values at some sites (Fig. 1). However, importantly, neither the nodule number- δ^{15} N relationship, nor δ^{15} N values themselves, were significantly influenced by Australian inoculum addition, but δ^{15} N values did differ across sites (Fig. 1). Therefore, these differences in seedling performance we observed are likely due to differences in soil-specific abiotic properties, such as differences in nutrient levels or water-holding capacity and/or differences in biotic conditions such as pathogen loads (Thrall et al. 2007). While we did not test for differences in these soil characteristics between our study sites, previous research illustrates that these are likely to be present in our soils. Keet et al. (2021) sampled soils throughout the CCR and found pH, bioavailable inorganic phosphorus, total carbon, nitrate-nitrogen (NO_3^{-}) , ammonium-nitrogen (NH_4^{+}) and total available nitrogen to differ significantly among all sampled sites. Importantly, Keet et al.'s (2021) study was conducted at similar spatial scales to ours and at sites near ours. Such strong spatial turnover in soil abiotic conditions is characteristic of CCR habitats (Linder 2003, 2005) and likely impacts both soil microbial (e.g., Keet et al. 2019) and plant (see Ellis et al. 2014) communities. The overall effects of soil type on plant performance that we observed here, therefore, likely reflect major differences in soil abiotic conditions and their knock-on effects on soil microbial communities, between sites. Also, while we did not quantify differences in nitrogen isotopic fractionation between soils, such differences may explain the differences in $\delta^{15}N$ values we observed for both legume species between sites.

While we cannot completely exclude the possibility that cross-contamination explains the dominance of the same Bradyrhizobium strains in A. saligna root nodules of seedlings grown in inoculated and uninoculated soils, several considerations suggest that this is an unlikely explanation. Firstly, there was a significant overall inoculation effect for A. saligna seedlings for many performance measurements. This was never the case for P. pinnata seedlings. Secondly, stringent protocols to minimise cross-contamination were put in place during soil collection and processing, inoculation applications and the glasshouse experiment (watering, etc.). Furthermore, Keet et al. (2017) sequenced root nodule communities from 19 different Australian Acacia species (including A. saligna) sampled across a wide geographic range (up to 900 km apart) in South Africa. They used the same DNA barcode as we did and found that all acacias shared a few, but highly abundant, Bradyrhizobium OTUs. The most dominant OTU identified by them, JHK OTU1, comprising 49% of their 98,000 sequence reads, is also the most dominant Bradyrhizobium strain in this study, SW OTU1 (Fig. 4). More recently, Le Roux et al. (2018) characterised rhizobial communities in acacia-invaded (by six different species, including *A. saligna*) and uninvaded CCR soils. They found that dense acacia stands homogenised rhizobial community structure and enriched soils for Bradyrhizobium strains. The most dominant Bradyrhizobium OTU identified by Le Roux et al. (2018) from acacia-invaded soils was JLR OTU1, comprising almost 10% of the 99,600 sequence reads they obtained, which corresponds to strains previously isolated from acacias in Australia (UH_MG accessions; Urbina and Klock, unpublished) and Keet et al.'s (2017) JHK OTU1, and thus our SW OTU1 (Fig. 4). Additionally, both our Rustenberg Winery and Vergelegen Wine Farm sites were also previously sampled by Le Roux et al. (2018). These authors found relative abundances of Bradyrhizobium strains isolated from paired invaded and uninvaded areas at these two sites to be similar (Le Roux et al. 2018). Together with the known history of cointroduction of Australian Bradyrhizobium and acacias to South Africa (Ndlovu et al. 2013; Warrington et al. 2019), these findings strongly suggest that the most parsimonious explanation for the dominance of the same Australian Bradyrhizobium in A. saligna nodules between our treatments is that they are, in fact, already established and widespread in CCR soils.

Conclusion

This study adds to a growing body of evidence suggesting that rhizobial mutualist availability is no longer a major limiting factor for acacia invasion (see Wandrag et al. 2020 and references therein) as exotic Australian *Bradyrhizobium* strains appear to be established within pristine CCR soils (Ndlovu et al. 2013; Warrington et al. 2019). Additionally, the one novel *Mesorhizobium* association for *A. saligna* in this study did not hamper seedling performance, and similar associations with Mesorhizobium strains have been previously documented (Amrani et al. 2010; Boukhatem et al. 2012; Crisóstomo et al. 2013). Australian acacias have the ability to utilise a few (often co-introduced) Bradyrhizobium strains interchangeably (Wandrag et al. 2013; Keet et al. 2017; Warrington et al. 2019). Therefore, co-introduction of effective rhizobial partners and mutualist promiscuity are not mutually exclusive but may act synergistically to enhance acacia colonisation success and invasiveness. Consequently, the presence of Australian bradyrhizobia in pristine CCR soils suggests that this region is highly susceptible to further acacia invasion with potentially detrimental above- and belowground biodiversity impacts. The CCR is home to a high diversity of rhizobia (Le Maitre et al. 2011; Sprent et al. 2017; Dludlu et al. 2018a) which is tightly linked to legume community composition (Slabbert et al. 2010; Lemaire et al. 2015; Le Roux et al. 2016). While there were no notable negative impacts of exotic bradyrhizobia on the Mesorhizobium strains identified in this study, the same may not be true for other rhizobial genera or their legume hosts (Le Roux et al. 2016). Our results suggest that mutualisms with belowground microbiota can contribute more towards habitat invasibility than previously thought.

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Supplementary material I

Supplementary materials

Authors: Staci Warrington, Allan G. Ellis, Jan-Hendrik Keet, Johannes J. Le Roux Data type: docx

- Explanation note: Electronic Supplementary Materials (ESM1, ESM2). Tables S1–S9. Figures S1–S4.
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RESEARCH ARTICLE



Network-based analysis reveals differences in plant assembly between the native and the invaded ranges

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Abstract

Associated with the introduction of alien species in a new area, interactions with other native species within the recipient community occur, reshaping the original community and resulting in a unique assemblage. Yet, the differences in community assemblage between native and invaded ranges remain unclear. Mediterranean grasslands provide an excellent scenario to study community assembly following transcontinental naturalisation of plant species. Here, we compared the community resemblance of plant communities in Mediterranean grasslands from both the native (Spain) and invaded (Chile) ranges. We used a novel approach, based on network analysis applied to co-occurrence analysis in plant communities, allowing us to study the co-existence of native and alien species in central Chile. This useful methodology is presented as a step forward in invasion ecology studies and conservation strategies. We found that community structure differed between the native and the invaded range, with alien species displaying a higher number of connections and, therefore, acting as keystones to sustain the structure within the invaded community. Alien species acting like keystones within the Chilean grassland communities might exacerbate the threat posed by biological invasions for the native biodiversity assets. Controlling the spread of the alien species identified here as keystones should help managing potential invasion in surrounding areas. Network analyses is a free, easy-to-implement and straightforward visual tool that can be widely used to reveal shifts in native communities and elucidate the role of multiple invaders into communities.

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Keywords

Alien species, Chile, community assembly, co-occurrence, invasion ecology, Mediterranean grasslands, network analysis, Spain, transcontinental naturalisation

Introduction

Associated with anthropogenic activities, human beings have drastically increased the flow of plant species amongst distant regions worldwide, overcoming major biogeographical barriers and enhancing invasion events (Valladares et al. 2019). When species arrive in a new area, they undergo different adaptative processes related to the degree of disturbance, resource availability and species interactions (Silvertown 2004; Knapp and Kühn 2012). When these species, considered *alien*, become self-maintaining for long-lasting populations without human intervention, they are considered *naturalised* (Richardson et al. 2000; Pyšek et al. 2004). There are several hypotheses proposed to explain this naturalisation process which can be summarised in three non-exclusive main groups: propagule pressure, abiotic filters and biotic interactions (Catford et al. 2009). The combination and characteristics of these three hypotheses drive the naturalisation process to occur (Hastings 1996; Wilson et al. 2007). Understanding how naturalisation affects plant diversity in the invaded community has been a major issue for ecologists (Sutherland et al. 2013), as it entails changes in its assembly and dynamics (Heger and Trepl 2003; Cramer et al. 2008).

Community assembly is a deterministic phenomenon in which a regional pool of species interacts to form local communities that are ultimately influenced by stochastic events, migration, dispersal, abiotic factors, biotic interactions and evolutionary and biogeographic processes (Ricklefs 1987; HilleRisLambers et al. 2012). Along the assembly process, the species either segregate because of competitive processes, aggregate due to facilitation and tolerance or are randomly distributed (Ricklefs 1987; Götzenberger et al. 2012), therefore displaying a particular spatial structure within the community (Ulrich 2004). For alien plant species, comparing assembly patterns between the native and the invaded ranges, can provide valuable information not only about the ecological factors underlying the community assembly (Hortal et al. 2012), but also about unravelling what determines invasion success (Figueroa et al. 2004). Plenty of literature compares abundance between native and invaded ranges and most of them agree that, when both ranges share a vast number of species and environmental conditions, the abundance of alien species is expected to be similar in both, perchance being an indicator of invasion success (Firn et al. 2011; Parker et al. 2013; Colautti et al. 2014; Pearson et al. 2018). Furthermore, some studies have already compared the community composition between Mediterranean grasslands in the native and invaded ranges (for example, Leiva et al. 1997; Martín-Forés et al. 2014), but there are very few that compare their assembly (de Miguel et al. 2016; Galán Diaz et al. 2020).

Network analysis is a novel approach that has become a potent tool to study microbiology communities (Barberán et al. 2012; Chun et al. 2020) and food webs (Bauer et al. 2010). Network analysis can also be applied to study community assemblage (Girvan and Newman 2002; Proulx et al. 2005; Fortunato 2010; Calatayud et al. 2020); however, as far as we are aware, it has yet been unused to compare co-occurrence patterns in plant communities. Network analysis, applied to patterns of flora co-occurrence, can unravel new insights into interaction networks. This will complement the information on naturalisation processes provided by more traditional analytical methods as richness and diversity indexes (Steele et al. 2011; Williams et al. 2014; Ma et al. 2016). In addition, network analysis, applied to invaded communities, elucidates the role of successful invaders by identifying species acting as keystones in the community structure and assemblage (i.e. those species that fulfil a key role within a community or those involved in a significant number of interactions; Estrada 2007; Bauer et al. 2010; Martín González et al. 2010; Eiler et al. 2012).

The dynamics and stability of complex networks are directly related to their structure, where not all the nodes, in this case corresponding to species, assume the same relevance. When nodes with high importance are subsequently removed, the network structure collapses faster than when random nodes are removed (Albert et al. 2000; Memmott et al. 2004). The importance of a node can be quantified by centrality measures, like "betweenness centrality", which provides information on how the node connects different parts of the network (Freeman 1978; Newman 2003). Thus, the betweenness centrality score applied to plant communities helps identify the contribution of each species to the cohesion of the network (Newman 2003).

The Mediterranean-climate regions constitute an excellent scenario for assessing the questions of plant transcontinental naturalisations, as they share similar climatic conditions, but maintain considerable geographic distance amongst them (Guerin et al. 2014, 2018; Martín-Forés 2017; Casado et al. 2018). Concretely, the grasslands of the Mediterranean Basin and the Chilean Mediterranean Region are ideal candidates to explore how species assemble once they become naturalised. Both ranges have shared an historical-cultural legacy since the Spanish arrival in the 16th century. This resulted in an asymmetry in the direction of introduction of species which occurred mainly from the Mediterranean Basin to central Chile and only occasionally in the opposite direction (Casado et al. 2018). Such introduction of alien species from Spain to Chile took place in a sequential and relatively independent process (Groves and Di Castri 1991; Martín-Forés et al. 2012; Casado et al. 2015; Martín-Forés 2017).

Spatial patterns as a result of the co-existence between native and alien species in central Chile have recently been studied through null models by de Miguel et al. (2016). In this study, the authors conclude Chilean grasslands maintain a similar segregated spatial pattern to the Spanish ones. It seems that, in spite of the relatively recent introduction of alien species in Chile, native and alien species have assembled, establishing associations different from random amongst them. Surprisingly, a longer co-existence time between species in Spanish communities was not reflected in a different spatial pattern than in Chile. However, there is a knowledge gap regarding the differences in the internal structure (e.g. nodes, "betweenness centrality" score, amongst other parameters) of plant communities between Spain and Chile.

This paper is part of a larger study in which we targeted all the steps of the naturalisation process. We targeted the flows of species introductions amongst the Mediterranean regions (Casado et al. 2018) and the state-of-the-art of the species introductions in the Mediterranean Region of Chile (Martín-Forés et al. 2012). Regarding community assembly, we studied abiotic and biotic factors, filtering the community in both the native and the invaded range (Martín-Forés et al. 2015) and explored co-incident changes that could be driving such naturalisation processes (Casado et al. 2015). We also studied how co-occurrence amongst the species within each community differed in both ranges (de Miguel et al. 2016) and, within the invaded range, how plant communities evolved over time through natural succession (Martín-Forés et al. 2016). Besides, for three alien species, we studied trait differences displayed between both ranges (Martín-Forés et al. 2017, 2018a, b).

In this work, we carried out network-based analysis to compare community structure and species co-occurrence between plant communities in the native and invaded ranges. We hypothesised that the pool of species that co-exist in the native range, once they have naturalised, will become organised similarly within the invaded community. Therefore, here we: i) assess the similarity between the Chilean and Spanish communities by comparing their floristic composition and abundance, as well as how alien species associate with each other and with native species and ii) elucidate how the alien species pool has integrated and assembled with the native flora in the invaded range by applying network analysis.

Methodology

Study area

The study area is located in the centre-west of Spain (40°13'N to 37°51'N and from 4°23'W to 7°02'W) and central Chile (32°35'S to 37°00'S and from 70°46'W to 72°35'W) (Fig. 1). Both countries share similar land management and physiognomy, based on grasslands. In Spain, this is a historical anthropogenic landscape that transformed woodlands into pseudo-savannah (Le Houerou 1981; López Sáez et al. 2007) where holm oaks (*Quercus ilex subsp. ballota*) and cork oaks (*Quercus suber*) are accompanied by herbaceous species, usually winter annuals (Joffre et al. 1999; Marañón et al. 2009). Meanwhile, in Chile, the anthropogenic landscape is relatively young, associated with the Spanish arrival in the 16th century (Aronson et al. 1998; Figueroa and Jaksic 2004). As a consequence, the pre-colonisation scrub and forest vegetation (Di Castri et al. 1981) was transformed into a landscape of scattered *Acacia caven* mixed with alien and native annual species (Aronson et al. 1998; del Pozo et al. 2006).



Figure 1. Map of the study areas Chile and Spain, sampling sites as points.

The agrarian practices introduced in central Chile involved a significant flow of organisms between Spain and Chile with the implementation of agriculture and grazing practices. This flow has persisted over time, possibly taking place unintentionally associated with human transport (Groves and Di Castri 1991; Ovalle et al. 2015; Martín-Forés 2017; Casado et al. 2018). Due to the history of land use and disturbance of Chile's grasslands, almost half of its vegetation is composed of alien species, with Eurasia and the Mediterranean Basin being the main donors (Casado et al., 2018). The most representative families in the Chilean grasslands (Poaceae, Asteraceae and Fabaceae) are also the most dominant in the Spanish grasslands. Not only qualitatively, but also quantitatively, those systems have similarities: amongst the ten most abundant species in Chilean grasslands, 90% are native Spanish herbaceous species that became naturalised in Chile; similarly, amongst the ten most abundant species of Spanish grasslands, 90% are common with Chile and, therefore, successful colonisers in Chile (Martín-Forés et al. 2012).

Regarding abiotic conditions, both areas have mean annual temperatures ranging from 14.5 °C to 16.9 °C in Chile and from 13.1 °C to 17.0 °C in Spain. The total annual precipitation of the sites ranges from 468 to 1030 mm in Spain and from 303 to 1168 mm in Chile. The summer drought period, characteristic of Mediterraneanclimate, is more intense in Chile than in Spain. Both systems have slightly acid soils from igneous or metamorphic rocks (Martín-Forés et al. 2015; de Miguel et al. 2016).

Data origin

The data utilised in this paper have been obtained in the previous study by Martín-Forés et al. (2015). In that study, vegetation surveys were carried out in both Medi-

terranean-climate regions described above (see study area). In both countries, 15 sites were selected to cover the range of climatic conditions found in Mediterranean-type regions (Fig. 1; see Martín-Forés et al. 2015 for methodological details regarding the climatic gradients considered). The study system in all sites had a similar physiognomy, opened savannah-like woodlands with only a few scattered trees and similar values of woody cover. Land-use was also similar in systems of both countries, mainly extensive grazing by cattle and sheep (Ovalle et al. 2015). Locally, we observed some differences in the livestock load that varied between one to two sheep per hectare in Chile and between one to three sheep per hectare in Spain, possibly related to the greater or lesser seasonal heterogeneity of the herbaceous production potential (Ovalle et al. 1990). In each site, vegetation surveys were carried out in twelve 50×50 cm quadrats that were randomly distributed in open areas to avoid tree influence (Marañón et al. 2009; De Miguel et al. 2013). Therefore, 180 quadrats were surveyed for each country in 2011, in which we scored species richness and abundance, estimated as presence within the twelve quadrats within each site. In the analysis, only herbaceous species were considered, as naturalisation success depends on plant life form (Tecco et al. 2010) and around 90% of alien species are herbaceous (Fuentes et al. 2013).

A total of 262 species were identified between both countries (190 species appeared in Spain and 132 appeared in Chile) and classified into four groups: i) *alien species*, those native to Spain that have been naturalised in Chile (111 species); ii) *Spain exclusive species*, which includes native and alien non-Chilean species, which only occur in Spain (91 species); iii) *Chilean native species*, which are native to Chile and do not occur in Spain (55 species); iv) *other species*, those non-included species in the previous groups, which are Chilean native species that occur in Spain and native species). We did not include the group *other species* in the analysis, as the aim here was to compare alien species in both ranges. Hence, in this study, we have worked with three sets of species: *alien species*, *Spanish exclusive species* and *Chilean native species*.

Data analysis

The data obtained during the surveys were organised in two datasets: i) a presenceabsence dataset that contained this information for every species in each of the 30 sites (15 sites per country) and ii) a dataset that included abundance information from 0 to 12 quadrants of each specie for all of the 30 sites.

To analyse the community resemblance between Spain (native range) and Chile (invaded range), we made a Non-metric Multidimensional Scaling (nMDS), based on the Bray-Curtis dissimilarity matrix with the abundance data of all species and only with alien species. To compare the species co-occurrence patterns between the native and invaded ranges, we followed the probabilistic model developed by Veech (2013). This model analyses the probability that two species co-occur at a lower or greater frequency than the one observed without relying on randomisations. When two species co-occur at a greater frequency than that expected at random, they are considered to have a positive association. On the contrary, if this frequency is less than expected at random, they are considered to have a negative association. We analysed the presence-absence data of species for each country. In both ranges, we analysed two subsets of species: i) *all species* (i.e. alien plus exclusive in Spain and alien plus native in Chile) and ii) *alien* species on their own. Notice that, in both ranges, the dataset of *alien* species is the same, but with different species abundances.

For the network analysis, we constructed four networks with the co-occurrence analysis results with *all* the species: one for each range and, within each range, one for each type of association (positive or negative). In network analysis terms, the species are considered nodes and the associations are considered edges. For each network, different parameters were calculated: density, "assortativity" coefficient and assortativity degree. Density represents the ratio between the number of connections existing in the network and the number of possible connections; it ranges from 0 to 1 and gives information on how interconnected the network is (in ecological theory, it is also known as connectivity). The assortativity coefficient measures the selective linking between nodes and ranges from -1 to +1. Positive assortativity values indicate that nodes of a certain species group connect to other nodes of the same species' group (i.e. alien species, native species or exclusive species), while negative values indicate that nodes tend to connect with nodes of a different species group. The assortativity degree measures whether nodes with a high degree (i.e. level of connection to other nodes) connect to other nodes with a high degree, ranging from -1 to +1. Positive assortativity degree indicates well-connected nodes tend to connect with other well-connected nodes, whereas negative values indicate that well-connected nodes tend to connect to poorly connected nodes, giving information about the robustness of the network. Additionally, we calculated the betweenness score for each species in the network, which is defined by the number of paths through a node and gives information about the importance of the species in the community structure. In this sense, nodes with high values of betweenness connect areas of the network that otherwise would be unconnected, possibly acting as keystones (for more information see Newman 2003; Fortunato 2010; Noldus and Mieghem 2014). In this work, a "betweenness" score above 0.1 was arbitrarily considered as the cut-off point to compare ranges.

We carried out all analysis with R software 3.6 version (R Core Team 2015) using the *vegan* package (Oksanen et al. 2020) for the nMDS, the *cooccur* package (Griffith et al. 2016) for the co-occurrence analysis and the *igraph* package (Csardi and Nepusz 2006) for the network analysis and visualisation using the functions: *betweenness, as-sortativity, assortativity_degree* and *graph.data.frame*.

Results

The nMDS analysis used to compare the resemblance between communities of each range showed a great differentiation between the native and the invaded ranges when taking into account all species (Fig. 2a) and alien species (Fig. 2b). The 15 sites within each range appeared grouped together without overlapping between ranges in both nMDS. Both ranges had a major dispersion, caused by the community differences amongst the surveyed sites. The floristic composition differed slightly more amongst the sites in the invaded range (i.e. appeared more dispersed in the nMDS) than amongst those in the native one.

Regarding the co-occurrence results (Table 1), when all species were considered, in both ranges, the percentage of random associations is above 95%. If only alien species were considered, in the native and invaded range, the percentage of negative associations is 98.2 and 93.24, respectively. Meanwhile, the percentage of non-random associations within all species in the invaded range represents twice the value for that in the native range. If only alien species were analysed, this invaded value rises to 3.75 times the native value. When contrasting the type of associations, both ranges had more positive associations than negative: 54% of the non-random associations were positive in the native range, while in the invaded range, it is 53%. When only the alien species were compared, in the native range, 57% were positive associations compared to the 64% of the invaded range.

After the co-occurrence analysis, we applied the network analysis to those results to analyse the four networks (i.e. Spanish positive and negative associations networks and Chilean positive and negative associations networks). The resultant co-occurrence network plots for positive interactions (Fig. 3) showed that, in the native range, there is a large network formed by 62 species (a), a smaller network consisting of four species (b) and three isolated pairs (Fig. 3A). Meanwhile, in the invaded range, we observed a large network of 35 species (c), a medium-sized network of 12 species (d) and three isolated pairs (Fig. 3B). In both ranges, the larger networks seemed to be connected by alien species: *Moenchia erecta* (L.) P.Gaertn., B.Mey. & Scherb. in the native range (species 117 in Fig. 3A) and *Briza maxima* L. and *Tolpis barbata* (L.) Gaertn. in the invaded range (species 10 and 146, respectively in Fig. 3B).



Figure 2. NMDS results for the sampling sites. Community resemblance between Spain (native range) and Chile (invaded range) was studied through non-metric Multidimensional Scaling (nMDS), based on the Bray-Curtis dissimilarity matrix with the abundance data. The graphic representation for the 30 sites surveyed considers: **a** all the species and **b** the pool of alien species. Sites in the native range are represented with blue dots, while sites in the invaded range are in orange. A polygon has been outlined connecting the sites of each country to improve the visualisation.

Table 1. Co-occurrence results for each range. Co-occurrence results of non-random associations for all species in Spain (native range), all species in Chile (invaded range), alien species in Spain and alien species in Chile.

		Non-random associations		
Country	Species group	%	positive	negative
Spain	All	2.29	102 (54%)	85 (46%)
Chile	All	4.84	90 (53%)	80 (47%)
Spain	Alien	1.80	31 (57%)	23 (43%)
Chile	Alien	6.76	57 (64%)	32 (36%)



Figure 3. Visualisation of the co-occurrence network for both ranges. Green lines connect pairs of species that have a positive association. The numbers are the species ID and each of the three species types have a separate enumeration for each country (see Suppl. material 1: Table S1). To improve visualisation, only species involved in non-random positive associations are represented. **A** Positive association network of all species in the native range (Spain), where two networks have been identified, one large (a) and one small (b). **B** Positive association network of all species in the invaded range (Chile), where two networks have been identified, one large (c) and one medium (d).

Focusing on the type of species involved in each network, in the native range, both exclusive and alien species were homogeneously intermingled in the large network, whereas only alien species aggregated in the small network and in pairs. Both native and alien species were present in the invaded area within the large network. On the other hand, Chilean native species remained mostly in the outer zone of the network, except for *Carex bracteosa* Schwein., *Cicendia quadrangularis* (Lam.) Griseb., *Juncus pallescens* Wahlenb. and *Plantago firma* Kunze ex Walp. (species 24, 32, 70 and 116, respectively in Fig. 3B). In the same way, the medium-sized network was formed almost completely by alien species; only the Chilean native species *Cladanthus mixtus* (L.) Oberpr. & Vogt. (species 31 in Fig. 3B) was connected by one association. Regarding the three independent pairs, two of them were formed only by Chilean native species and one by one native and one alien species.



Figure 4. Visualisation of the co-occurrence network for both ranges.. Red lines connect pairs of species that have a negative association. The numbers are the species ID and each of the three types of species have a separate enumeration for each country (see Suppl. material 1: Table S1). To improve visualisation, only species involved in non-random negative associations are represented. **A** Negative association network of all species in the native range (Spain), where three networks have been identified, one large (a) and two smaller ones (b, c). **B** Negative association network of all species of the invaded range (Chile), where two networks have been identified, a large one (d) and a smaller one (e).

The co-occurrences network plots for negative associations (Fig. 4) revealed that, in the native range, there is a large network formed by 53 species (a), two smaller networks formed by 4 (b) and 5 (c) species, two trios and five isolated pairs (Fig. 4A). Meantime, in the invaded range (Fig. 4B), we observed a large network of 41 species (d), a smaller network of 5 species (e), one trio and two isolated pairs. Contrary to the network visualisation for positive associations, it was challenging to distinguish the species acting as main connectors in the larger negative networks. Focusing on the groups of species implied in each network, in both ranges, native and invaded, both alien and exclusive or Chilean native species intermingled evenly in the larger networks.

		0 /1	1	0
Association sign	Range	Density	Assortativity coef-	Assortativity deg

Table 2. Network analysis results for each range and type of association (positive and negative).

Association sign	Kange	Density	Assortativity coef-	Assortativity degree
			ficient	
Positive	Native (Spain)	0.04	0.06	0.06
Positive	Invaded (Chile)	0.07	0.21	0.10
Negative	Native (Spain)	0.03	0.01	0.11
Negative	Invaded (Chile)	0.06	-0.10	0.02

Table 3. Species with a betweenness score	greater than 0.10 for	r each of the four net	works. Note that the
0.1 score is an arbitrary cut-off point.			

Positive association network in the native range (Spain)					
ID	Species	Туре	Betweenness score	Growth form	
149	Romulea bulbocodium	Spain exclusive	0.3347	Geophyte	
117	Moenchia erecta	Alien	0.3330	Terophyte	
127	Parentucellia latifolia	Alien	0.3315	Terophyte	
190	Trifolium tomentosum	Alien	0.2633	Terophyte	
35	Carlina racemosa	Spain exclusive	0.2513	Terophyte	
124	Ornithopus perpusillus	Spain exclusive	0.2381	Terophyte	
16	Aphanes microcarpa	Spain exclusive	0.1738	Terophyte	
3	Agrostis castellana	Alien	0.1618	Hemicryptophyte	
93	Jasione montana	Spain exclusive	0.1445	Terophyte	
67	Euphorbia exigua	Spain exclusive	0.1410	Terophyte	
125	Ornithopus pinnatus	Alien	0.1081	Terophyte	
Positive association network in the invaded range (Chile)					
ID	Species	Туре	Betweenness score		
108	Petrorhagia prolifera	Alien	0.1950	Terophyte	
10	Briza maxima	Alien	0.1568	Terophyte	
146	Tolpis barbata	Alien	0.1436	Terophyte	
Negative association network in the native range (Spain)					
ID	Species	Туре	Betweenness score		
93	Jasione montana	Spain exclusive	0.1904	Terophyte	
16	Aphanes microcarpa	Spain exclusive	0.1467	Terophyte	
35	Carlina racemosa	Spain exclusive	0.1170	Terophyte	
Negative association network in the invaded range (Chile)					
ID	Species	Туре	Betweenness score		
55	Galium murale	Alien	0.2405	Terophyte	
4	Anagallis arvensis	Alien	0.2170	Terophyte	
48	Erodium botrys	Alien	0.11901	Terophyte	

Focusing on the positive associations from the network analysis, the invaded range had superior values in the three calculated parameters (density, assortativity coefficient and assortativity degree), the assortativity coefficient values (3.5 times higher in the invaded range) being outstanding. Regarding the negative associations from the network analysis, the invaded range had a density value double that for the native range; however, the assortativity degree was 5.5 times lower. The assortativity coefficient of the invaded range stood out as the only negative value within the networks, indicating that nodes tend to connect with nodes of different type (Table 2). This means that the alien species tend to form negative associations with native species and vice versa.

To obtain information on the importance of species in the community structure, the betweenness score was calculated. When observing the positive association network, in the native range, there are several species with betweenness values higher than 0.1, five of them are alien species and six are exclusive to Spain. In the invaded range, there are only three species, all of them alien. When looking at the negative association network, only three species are above 0.1 in both ranges. In the native range, these species are all exclusive to Spain, while in the invaded range, there are, once again, alien species.

Discussion

Community assembly of Mediterranean herbaceous species differs between Spain and Chile with little resemblance amongst community structures in the native and invaded ranges. Regarding community structure in the invaded range, most of the species that establish non-random associations correspond to alien species, which surprisingly act as keystones within the community. In the invaded range, the assembled community has higher network connectivity. Moreover, alien species in the invaded area tend to assemble more amongst them displacing native species to the edges of the network.

Although there were more alien than native species in the invaded study area (Martín-Forés et al. 2012), both ranges showed a contrasting resemblance, differing in their community structure when the abundance of all species was taken into account (Fig. 2a). This pattern persisted when comparing alien species common to both countries, suggesting that alien species are particularly significant in shaping communities in the invaded range (Fig. 2b). After the introduction related to the Spanish colonisation in the 16th century (Martín-Forés et al. 2015), climate, amongst other abiotic conditions, strongly influenced the establishment of alien species in the invaded range, as it also does on the community assembly in the native range (Figueroa et al. 2004; de Miguel et al. 2016; Rota et al. 2017). Despite the climatic similarity, the higher water deficit in the invaded area (up to 8 months in the Mediterranean Region of Central Chile versus 4 months in Spain; Martín-Forés et al. (2015)) and the local differences in livestock management between both ranges (del Pozo et al. 2006; Casado et al. 2015), may be influencing the heterogeneity found within the two countries. Differences between Chilean communities at the regional scale may be due to the regional precipitation gradient that conditions water availability (Martín-Forés et al. 2015) (Fig. 2).

Our results showed that the associations of the alien species differed within the plant communities in both native and the invaded ranges. This suggests that the associations of alien species in the invaded area is an inherent property of being away from their native range, rather than a property of the species itself (Stotz et al. 2020). For example, alien species can tend to aggregate amongst them in the invaded range and they have been found to establish preferably in species-poor places with greater levels of disturbance and higher nutrient availability, biomass and competition (Stotz et al. 2020). We found that, in both ranges, less than half of the total number of species are involved in non-random associations. The non-random pattern of species assemblage displayed by the communities in both ranges (i.e. the higher or lower ratio of co-occurrence than that expected from random; Griffith et al. 2016), suggested that these communities are structured by deterministic processes instead of stochastic ones (Krasnov et al. 2010). It seems that the co-existence of species for longer periods of time tended to randomise the associations, as in Spain, the percentage of non-random association was smaller than in Chile. This is in slight contrast to the results obtained by de Miguel et al. (2016), in which they detected that, in Chile, the spatial segregation of species was similar to Spain. This demonstrates that a longer co-existence time does not determine a greater spatial segregation of species. Conventional knowledge indicates that species interactions, especially competition, are the main forces shaping community structure, but habitat heterogeneity is also a source of non-random spatial distributions of species (Ulrich 2004; Kraft et al. 2015). Even though both areas harbour similar climatic conditions, other regional factors, such as water availability (Acosta et al. 2008), soil fertility (Holmgren et al. 2000; Price et al. 2014) and fire regimen (Brotons et al. 2013) under the combined effect of grazing, could affect this association processes revealing a divergence in the communities (Martín-Forés et al. 2012; Peco et al. 2017).

The network analysis applied to the co-occurrence patterns resulted in a visual output that revealed meaningful structural information (Newman 2003; Fortunato 2010). At the community level, communities in both the native and invaded ranges have very poorly connected co-occurrence networks, both positively and negatively, as evidenced by the near-zero density (Newman 2003). This supports the idea that. in both ranges, grassland communities are primarily driven by abiotic factors rather than by biotic relationships between species, whether native or alien (Casado et al. 2015; Martín-Forés et al. 2015). The assortativity degree results manifested similar tendencies in both countries, where species slightly tend to connect to other well-connected species (Fig. 3). This is coherent with the typical properties of biological networks, where it is common to find large clusters of nodes with a high level of internal connections, but weakly connected to other clusters, known as the modularity of a network (Sah et al. 2014). This modularity in biological networks is known to promote cohesion and contain disturbances, as species in one module are more tightly linked to each other than to species in other modules (Olesen et al. 2007; Stouffer and Bascompte 2011). This structure perhaps is an additional strategy of the Mediterranean grasslands that allows them to be particularly resilient systems despite the constant disturbances that characterise the Mediterranean Region, such as fire, thermal oscillation, irregular

rainfall distributions between years and the summer drought (Henkin et al. 2010; Acosta and Pineda 2012).

Observing the network structure, in the native range, alien species integrate with the rest of the species in a large network, as the "alien" grouping in the native range is an artificial group that allows the comparison with the invaded range (see Figure 3A). In the invaded range, some alien species appear to be connected to each other and native species only participate partially in these networks, without constructing independent networks of their own. The fact that the percentage of associations between alien species was almost four times higher in the invaded range than in native range (Table 1) indicates that, in the invaded area (Chile), the alien species tend to be more connected. This result could be due to shared habitat preferences amongst alien species, as found out by the study conducted in grasslands by Stotz et al. (2020). Furthermore, in the invaded range, amongst alien species, positive associations were higher and negative associations lower than in the area of origin, similar to the findings of Braga et al. (2018) in a large-scale study. This suggests that facilitation mechanisms are occurring between alien species in the invaded range, which could, in turn, lead to an "invasional meltdown", where a successful invader may trigger subsequent invasions, increasing their chance of survival and ecological impact (Simberloff and Von Holle 1999: but see Divíšek et al. 2018).

Preferences for associations that occur between species also vary between ranges and the results of the invaded area are striking. Only a group of species from the area of origin (Spain) has been able to remain in the invaded area (Chile) and naturalise (Martín-Forés et al. 2012), probably due to having similar naturalisation mechanisms. Once established, the alien species tend to positively associate amongst them and negatively with the native species, with the exemption of few native species that positively associate with the alien ones (Figs. 3b and 4b and Table 2). This could be due to facilitation mechanisms between alien species (Braga et al. 2018); similarly, the tolerance of plants to grazing pressure could be influencing their configuration (del Pozo et al. 2006).

The results of the network analysis are striking because when literature from other Mediterranean areas with the same source-invader relationship has compared the assemblage in terms of abundance, alien species assemble similarly in their source and recipient communities (Firn et al. 2011; Parker et al. 2013; Colautti et al. 2014; Pearson et al. 2018). In contrast, by applying this methodological approach, we can observe that alien species assemble very differently in the invaded area. It seems that the set of species that arrive in the invaded area of distribution reorganise themselves differently from the original community of which they formed part in the native area (Spain), coexisting with native species with which they show less affinity.

Concerning the species acting as keystones, alien species in the invaded range have an important role in the community structure. We expected Chilean native species would play a key role in Chilean communities, but our results suggest the opposite. In the invaded range, alien species showed the highest betweenness score, acting as a cornerstone for both positive and negative associations. In Chilean agroecosystems, eradication of alien species has not been a main management strategy (Root-Bernstein and Jaksic 2013), which could be the explanation for alien species developing key roles within the community. The implications for management strategies and conservation practices are, therefore, delicate. On the one hand, after a disturbance, alien species could preserve the rest of the community in a well-connected manner (but see Carmona et al. 2017), enhancing the resilience and functional persistence of ecosystems by strengthening the network interacting with native species (Aslan 2019). On the other hand, if left unmanaged, those alien species could displace the Chilean native flora. In addition, since the introduction and naturalisation of these key alien species are associated with grazing (Arroyo et al. 2000; Figueroa et al. 2004; Martín-Forés et al. 2012) and that the long-term stability of these grasslands depends on certain intensity and periodicity of human activities (del Pozo et al. 2006), any land-use changes could trigger their spread or regression. Further studies on elucidating this trend would be desirable.

Meanwhile, the alien species that play important roles in the invaded range do not do so in the native range. In the native range, a greater number of species were actively involved in shaping the network (i.e. displayed high betweenness), providing greater stability against disturbances, probably because these Spanish agro-ecosystems have been maintained over millennia (Acosta and Pineda 2012; Landi et al. 2018).

Although it is questionable whether theoretical models can accurately predict assembly patterns and how environmental factors and land uses might alter the effects of species interactions on species co-occurrence (Ulrich et al. 2017), unseen patterns have emerged. The next step may be to explore the networks in detail to understand spatio-temporal dynamics on a regional or patch scale and identify patterns that may depend on the scale of analysis (Smith et al. 2013; Escobedo et al. 2021). However, we considered processes at a small spatial scale (0.5 m²), because, in grasslands, at larger spatial scales, more positive correlations between native and alien species can be found, but they are driven by environmental heterogeneity instead of by biotic interactions, giving rise to misleading results (Davies et al. 2005). In addition, functional traits are known to play a key role in community assembly and invasiveness (Tecco et al. 2010; de Bello et al. 2012; HilleRisLambers et al. 2012; Thuiller et al. 2012; Colautti et al. 2014; Galán Díaz et al. 2021) and positive associations between rare species persist across taxon assemblages (Calatayud et al. 2020). Similarly, certain growth forms can be displaced by alien species in grassland communities (Guerin et al. 2019), therefore including species' trait syndromes in further studies of species co-occurrence would help unravelling insights of the assembly process. It should also be noted that our results may be conditioned by the short time period included in the analysis (one year) as the Mediterranean climate region suffers from a large inter-annual variation in plant community composition. This variation is due to the irregularity of the rainfall regime that encourage alternatively the abundance of more or less drought-tolerant species (Acosta et al. 2008; Peco et al. 2009; Rota et al. 2017). We recommend further studies to explore changes in community structure over time via resampling the same community after a certain period of time (in this case, the changes are more perceptible after 4-6 years according to Martín-Forés et al. 2016) and applying the methodology

described here. This will allow accounting for failure of establishment and invasion lag periods in the process of community assembly.

Our novel methodology, proposed in this paper, improves our understanding of how the naturalisation process affects community assembly and structure. Network analyses are a free, easy-to-implement and straightforward visual tool that can be widely used in community ecology, to unravel assembly patterns, which would enable the prediction of changes in ecosystem structure and functioning under different changing scenarios. Similarly, in studies of invasion ecology, it allows revealing shifts in native communities and elucidates the role of multiple invaders into communities.

Thus, the identification of negative and positive associations, as well as elucidating which species act as keystones, is presented as a step forward in invasion ecology studies and as a tool to help designing restoration and conservation strategies in socioecological systems (Root-Bernstein and Jaksic 2013). This is particularly relevant in areas where multiple environmental and social factors operate and intermingle, such as in the Mediterranean biome (Doblas-Miranda et al. 2017). Grazing systems in Mediterranean Regions are essential as sustainable and biodiversity-friendly food production systems of the future, amongst other ecosystem services, such as hydrological regulation and carbon sequestration (Acosta and Pineda 2012; Root-Bernstein et al. 2017), being absolutely critical in the actual context of global change (Van Kleunen et al. 2015; Cramer et al. 2018; IPCC 2018; Lionello and Scarascia 2018).

Conclusion

With this work, we have proved the utility of combining network analysis to co-occurrence analysis, as it has permitted the process of exploring complex sets of data and results in a very interesting way. Our results showed that the community assembly pattern differs between the native and the invaded ranges. Alien species that became naturalised in the invaded area re-assembled differently, tending to co-occur more amongst them and to avoid interaction with native species. Moreover, alien species acted like keystones in the communities of the invaded range. The key role displayed by alien species in Chilean communities could cause an invasional meltdown and threaten the native biodiversity. Conservation and managing strategies should focus on monitoring and controlling their potential spread and developing early detection strategies especially for the alien species identified as keystones.

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Supplementary material I

Table S1. Species ID for each country and type of species

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Data type: species data

- Explanation note: Species ID for each country, type of species and relative abundance. Note that each species has a different ID in each range.
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Modelling the likelihood of entry of marine non-indigenous species from internationally arriving vessels to maritime ports: a case study using New Zealand data

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Abstract

The establishment of marine non-indigenous species (NIS) in new locations can degrade environmental, socio-cultural, and economic values. Vessels arriving from international waters are the main pathway for the entry of marine NIS, via exposure due to ballast water discharge (hereafter, ballast discharge) and biofouling. We developed a systematic statistical likelihood-based methodology to investigate port-level marine NIS propagule pressure from ballast discharge and biofouling exposure using a combination of techniques, namely k-Nearest-Neighbour and random forest algorithms. Vessel characteristics and travel patterns were assessed as candidate predictors. For the ballast discharge analysis, the predictors used for model building were vessel type, dead weight tonnage, and the port of first arrival; the predictors used for the biofouling analysis were days since last antifouling paint, mean vessel speed, dead weight tonnage, and hull niche area. Propagule pressure for both pathways was calculated at a voyage, port and annual level, which were used to establish the relative entry score for each port. The model was applied to a case study for New Zealand. Biosecurity New Zealand has commissioned targeted marine surveillance at selected ports since 2002 to enable early detection of newly arrived marine NIS (Marine High-Risk Site Surveillance, MHRSS). The reported methodology was used to compare contemporary entry likelihoods between New Zealand ports. The results suggested that Tauranga now receives the highest volume of discharged ballast water and has the second most biofouling exposure compared to all other New Zealand ports. Auckland was predicted to receive the highest biofouling mass and was ranked tenth for ballast

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discharge exposure. Lyttelton, Napier, and New Plymouth also had a high relative ranking for these two pathways. The outputs from this study will inform the refinement of the MHRSS programme, facilitating continued early detection and cost-effective management to support New Zealand's wider marine biosecurity system. More generally, this paper develops an approach for using statistical models to estimate relative likelihoods of entry of marine NIS.

Keywords

ballast water, biofouling, biosecurity, marine ecosystems, non-indigenous species, vessels

Introduction

Marine ecosystems are vulnerable to the introduction of non-indigenous species (NIS) through human activities such as international trade and transportation, which have increased greatly in the last fifty years (Hulme 2009). International marine shipping, which contributes to 90% of world trade, is a leading source of introduction of many marine NIS (Hewitt et al. 2009; Hulme 2009; International Maritime Organization [IMO] 2017). Marine NIS have the potential to impact upon environmental, socio-cultural, and economic values. For example, in 1991, the outbreak of a virulent strain of cholera infected one million people and claimed thousands of lives and was linked to the discharge of ballast water in Peru (McCarthy and Khambaty 1994; Tauxe et al. 1995; Kolar and Lodge 2001). Therefore, it is essential to describe existing patterns of invasion and forecast future invasions for improving management of marine ecosystems (Drake and Lodge 2004).

The two main pathways associated with the spread of marine NIS via shipping are ballast water discharge (hereafter, ballast discharge) and biofouling exposure (Hewitt and Campbell 2010). Until recently, ballast water was thought to be the major contributor to marine NIS spread; however, the biofouling pathway has been assessed to be as great, if not a higher threat (Cordell et al. 2009; Hewitt and Campbell 2010; Bell et al. 2011). In New Zealand, it has been estimated that between 69–90% of established marine NIS are likely to have been introduced via biofouling, with ballast discharge being the second most important pathway (Cranfield et al. 1998; Kospartov et al. 2008). International and national policies have been implemented to minimise the spread of marine NIS through ballast water and biofouling, and New Zealand is currently the only country to have enacted mandatory biofouling regulations (Georgiades et al. 2020).

The use of predictive models can be a powerful tool for helping to understand the dynamics of invasion pathways through the exploration of factors that influence propagule pressure (Verling et al. 2005). Variables that influence propagule pressure can be classified as voyage properties (e.g., duration, source region, visited locations, arrival date or season), vessel characteristics (e.g., size, ballast water capacity, niche area type and extent) and behaviours (e.g., compliance with regulations), all of which influence

the likelihood of entry for both pathways (McGee et al. 2006; Lacoursière-Roussel et al. 2012; Minton et al. 2015).

Many studies have identified high-risk invasion pathways (Cope et al. 2015), global hotspots of invasions (Drake and Lodge 2004), possible source ports of invasion (Paini and Yemshanov 2012), and high-risk vessels arriving from international ports (Clarke et al. 2017). However, few studies have established how information about vessel traffic patterns and vessel characteristics for ballast water and biofouling pathways can be integrated to identify ports most likely to receive marine NIS. We demonstrate the use of an integrative modelling approach using the data available from New Zealand, to investigate propagule pressure differences among ports.

The Marine High-Risk Site Surveillance (**MHRSS**) Programme was established in 2002 to facilitate the early detection of selected marine NIS that were considered likely to impact on New Zealand's marine values. Currently, 11 ports and marinas are considered 'high-risk' for marine NIS entry and establishment (Seaward et al. 2015). The original site selection was based on proxy indicators using data from 1999, e.g., vessel visits and port habitat types (see Inglis 2001a, b, Inglis et al. 2006). Since the inception of this programme, changes in vessel movement patterns and behaviours are likely, and re-evaluation is appropriate. At the time of the study, the allocation of surveillance effort was identical to all ports except at Auckland, at which it was doubled.

The aim of this research is to develop a systematic statistical likelihood-based approach for predicting marine invasions. We investigate whether the ports and marinas currently surveyed as part of the MHRSS programme are most likely the locations where marine NIS will enter New Zealand with internationally arriving vessels via the ballast and biofouling pathways. We then assess whether the allocation of survey effort at each location should be modified based on these findings.

Methods

Datasets were provided by Biosecurity New Zealand (**BNZ**), the government agency responsible for New Zealand's biosecurity system. The main challenge in predicting the hazard presented by ballast discharge for the current case study is that contemporary ballast discharge records were not available. BNZ collected ballast discharge intention declaration data up until 2008, but risk analysts were concerned that the pattern and volume of transits may have changed substantially, hence the need to connect the historical and contemporary data described below.

The challenge in predicting the hazard due to biofouling exposure is to construct a model that permits prediction of the biofouling level based on easily captured vessel statistics. As described below, we used data from a historical biofouling study to construct this model.

For vessel ballast water and biofouling pathways we used two sets of historical and contemporary data as described below.

Historical data

Ballast discharge

A historical dataset of biofouling and ballast water declarations was compiled for vessels that visited New Zealand during 1998 to 2008, which contained records for 15,745 voyages by 2,607 vessels. This dataset contained information on the vessel's physical and voyage characteristics, and ballast discharge volumes detailed for each ballast tank. This dataset was examined to determine: (i) unique voyage and port visits by vessels and volumes of ballast discharged per vessel at each port, and (ii) management of the ballast and intention to discharge in New Zealand. The response variables used for ballast water modelling were the port of discharge and discharge volume per port. Vessel type, ballast capacity or Deadweight Tonnage (DWT), arrival port, and the intention of discharge were candidate predictor variables for estimating how much ballast water was discharged at each port. Arrival port is the first New Zealand port a vessel encounters in a voyage. Many voyages declared more than one port of ballast discharge, i.e., each journey has a first arrival port, but the vessel might visit other ports as part of the same voyage and discharge ballast. For example, a vessel's voyage to New Zealand may include visits to Whangarei, Tauranga and Auckland, with intended ballast discharge at Whangarei and Tauranga but not Auckland; here the recorded intended ballast discharge volumes for the first two ports (i.e., those at which ballast was discharged) were tallied. The historical data contained 15,628 unique voyages out of which 1,196 journeys had more than one port of ballast discharge. Out of 17,703 stops at ports of arrival, there were 6,287 incidents of reported ballast discharge. Note that the discharge may have presented an acceptable biosecurity risk, if the discharge were carried out in a regulated way, for example after open-ocean exchange. 'Intent to discharge' was not used as a predictor because there was poor agreement between the declared intention to discharge and the reported discharge volume. More information about this agreement can be found in the Suppl. material 1: Table S1 in the SM summarises the description of the variables used in the historical dataset for the ballast water analysis.

Biofouling

Biofouling on the submerged surfaces of 322 international merchant vessels was sampled by Inglis et al. (2010) upon arrival to New Zealand. This included 166 container and general cargo vessels, 49 passenger vessels, 37 roll-on roll-off (RoRo) / car carriers, 31 bulk carriers, 21refrigerated cargo ships, 12 tankers and 6 vessels of other miscellaneous class types. A standardised sampling design was used to collect samples of biofouling from the hull and external niche area surfaces of the vessels. A questionnaire administered to the vessel's master provided information on the design features of the vessel, hull maintenance record (e.g., age and type of antifouling coatings), and recent voyage history.

Data on the presence and mass of biofouling from 314 of the merchant vessels and associated data from the questionnaire were used to build the biofouling model. As the contemporary arrival data to which the model would predict contained only limited information on the recent voyage history of the vessels, the models were fitted using variables from the historic study that could be easily matched to contemporary information collected by BNZ. They included design specifications of the vessel (e.g., class type, design speed), vessel age, niche area, the age of antifouling coatings on arrival, and the maximum periods of inactivity (lay-up) in any location. For biofouling data, the main two response variables were biomass of biofouling per square metre and presence/absence of any biofouling. The latter had a value of 1 whenever biofouling was present, and zero if there were no biofouling. This binary variable was used to correct mass; that is, whenever mass had a zero value, but biofouling was present, a correction value of 0.001 g was added to the mass. Predictor variables in the historical biofouling data were the age of antifouling coating, vessel type, vessel age, vessel speed, season, niche, and maximum duration of lay-up. The description of the variables and their ranges are given in the Suppl. material 1: Table S2.

Data on contemporary shipping

Contemporary data sourced from BNZ on international vessel arrivals into New Zealand were a combination of two data sets. The first included data on vessel arrivals between January 2015 to December 2017, and was derived from mandatory pre-arrival documentation provided by the vessel master to BNZ (Ministry for Primary Industries 2019). These data were originally collected to aid in the tactical evaluation of biosecurity risks. The second data set included port-by-port arrivals by merchant vessels, including the ports of first arrival to New Zealand and coastwise voyages for the year 2016. These data were purchased from the Lloyd's Maritime Intelligence Unit, and used to verify the completeness of the BNZ data. Details of all data preparation for the analysis are provided in the Suppl. material 1 – part 1. The same contemporary data were used for both ballast water and biofouling analysis, but a different set of predictors were selected for each.

Model construction

The ballast discharge model was used to predict the total annual volume of ballast water discharged at each New Zealand port. The contemporary data lacked ballast discharge information, so we had to predict it from historical data. The models were built to predict ballast discharge at each port as a function of arrival port and ship characteristics.

The historical biofouling data from Inglis et al. (2010) were used to fit a model that predicted the presence and biomass of biofouling on vessels that arrived in New Zealand between 2015 and 2017. The total mass of biofouling on a vessel was estimated as the product of its estimated mass per unit niche area (i.e., density) and the total niche area

for the vessel. A model was constructed from historical data to predict the presence and per unit area of biofouling in niche areas of the vessels as a function of vessel characteristics such as the age of antifouling coatings on the vessel, and the frequency and duration of lay-up periods. As most biofouling occurs in niche areas (Inglis et al. 2010; Davidson et al. 2016), the total mass of biofouling on a vessel was estimated by calculating the total area of niches on each vessel using relationships developed by Moser et al. (2016) and scaling the predicted density for the entire vessel hull area. This model was then applied to the contemporary vessel data to predict port-level exposure to biofouling. Additional details about the modelling approach can be found in the Suppl. material 1 – part 3.

Ballast water

This section reports construction of an algorithm to predict the volume (and at which ports) a vessel will discharge their ballast water, given their vessel-level characteristics (e.g., type, DWT, Ballast capacity) and journey-level information. The complex aspect of this endeavour is that after entering New Zealand's waters, each vessel may visit more than one port, with the possibility of discharging ballast water at one or more of the ports visited. As a result of discharging at one or more ports, the variable of interest is structured as a vector for each vessel, so any algorithms investigated are required to take this vector-based outcome into account.

k-Nearest-Neighbours

An algorithm based on k-Nearest Neighbours (KNN, Fix and Hodges 1951) was developed to simultaneously predict which ports a vessel may visit and the subsequent discharge amount. For each journey in the contemporary data, this algorithm finds the k most similar journeys in the historical data based on some measure of similarity and uses the average ballast discharge from those journeys as a prediction of the discharge for the contemporary journey.

Denoted by X the n × p matrix of data used for model training (the training data is the historical data as described in the Suppl. material 1 – part 1), where n is the number of voyages (observations) and p is the number of fields (predictors) in the data; let Y be the corresponding n × v matrix of discharge volumes, where v is the number of ports. Let z_j be a vector of length p, containing the predictors for the jth voyage in the contemporary data, for which we wish to predict ballast discharge, \hat{y}_j , at each of the ports. KNN algorithm as applied to ballast discharge data can be explained as below:

1. Repeat for j = 1, ..., m, where m is the number of voyages in the contemporary data for which we wish to predict:

a) Calculate $D(z_i, X)$, the distance from z_j to each of the rows xi, i = 1, ..., n in the training matrix X.

b) Find S, the set of the k^{th} smallest distance in D (if there are ties, keep these in the set).

c) Calculate

$$\hat{y}_j = \frac{1}{|S|} \sum_{i \in S} y_i$$

i.e., the predicted discharge values are the average of the k^{th} nearest neighbours discharges.

- 2. For each year *t* in the contemporary data:
 - a) Find $S_{r(v)}$, the set of all voyages arriving at port *v* during year *t*.
 - b) Calculate

$$\hat{B}_{t[\nu]} = \sum_{i \in S_{t[\nu]}} \hat{y}_{t[j]}$$

i.e., $\ddot{B}_{t[v]}$ is the total predicted ballast discharge in port *v* during year *t*.

The KNN algorithm does not work for categorical predictors, as there is no defined distance metric for mixtures of categorical and numerical predictors. To allow for categorical predictors (such as vessel type), the KNN algorithm was implemented on numerical predictor matrices created within each level of a category. For example, to use vessel type and dead weight tonnage as predictors, the algorithm was run on the matrix X formed from the subset of voyages from each level of vessel type until all predictions were made.

The historical data were structured such that each row of the dataset represented a single event in a vessel's voyage. For example, one row may consist of the arrival event, with no discharge, and the next row a discharge event for the same vessel at a different port. It was important that if a vessel did not discharge in a port, then this non-event was not recorded. In order to have a complete vector response for each voyage, it was reasonably assumed that non-events resulted in a 0 discharge being recorded. An example of this transformation is provided in Suppl. material 1: Fig. S2 in the Suppl. material 1 – part 4.

Choice of predictors and number of nearest neighbours, k

The choice of which predictors to include, and how many nearest neighbours to use in the prediction was made using cross-validation. Given our prediction target of total ballast water volume at a port within a year, our cross-validation strategy was to leave out a single year at a time to form the testing set, with the remaining year's data forming the training set. A schematic of cross-validation used for KNN analysis is given in Suppl. material 1: Fig. S3 in the Suppl. material 1 – part 4.

Model validation

In order to provide an unbiased validation of the chosen model and help understand its operational characteristics, the data were split into two: model-fitting data comprising all voyages within the years 1999 to 2005 (inclusive) and validation data comprising all voyages within the years 2006 and 2007.

Prediction errors

To estimate the prediction error from the KNN algorithm, a bootstrap procedure was used. Voyages were randomly sampled with replacement from the training data, and the KNN algorithm fit on each bootstrap sample. The standard deviation (and mean/ median) of total discharge within a port was calculated from 100 bootstrap resampled values of total discharge.

Biofouling

Model selection

Several different modeling approaches were applied to the historical data in the process of model selection to estimate biofouling biomass for the contemporary dataset (Suppl. material 1 – part 6). Random forests provided the best model to obtain the prediction (Breiman 2001; Liaw and Wiener 2002; see Hastie et al. 2017 for a discussion) and we used quantile-regression random forests to obtain the distribution to represent the prediction uncertainty (Meinshausen 2017).

Random forests analysis was applied to the data because the algorithm is indifferent to the conditional distribution of the response variable, meaning that no special provisions need to be taken to allow for the heavy zero inflation. The random forest model explained about 15% of the variation in the biofouling mass, which was better than its alternatives. Quantile random forests was used to obtain journey-specific prediction distributions which could then be aggregated to obtain overall levels of uncertainty for the exposure of each port to biofouling for each year.

Model building for biofouling biomass

The likelihood of entry via biofouling was assumed to be proportional to the biofouling exposure at each port, each year. For a single vessel, this was assumed to be the predicted total mass of biofouling in the niche areas of the vessel (not including the hull as a niche area). However, the contemporary data did not contain biofouling mass measurements for each vessel but did contain measurements for each vessel's niche area in metres squared. To handle this, two models were constructed: a model to predict the biofouling mass per vessel as a function of several variables, and another model to predict the quantiles of the prediction distribution. The random forest model used the following continuous predictor variables: dry weight tonnage, days since last antifouling paint, niche area, and mean speed. Vessel type was assessed and was excluded from the models because the improvement of fit was insufficient.

To predict the density of biofouling for each vessel in the contemporary data, missing data were first handled using multivariate imputation via chained equations (Buuren and Groothuis-Oudshoorn 2010) to make five complete variant datasets, which were analysed in parallel. The random forest biofouling presence model was applied to each contemporary vessel movement, which gives an estimate of the quantum of biofouling. Then the prediction distributions were estimated using the quantile random forest. Once the density of biomass present on each vessel was predicted, it was multiplied by the niche area of the vessel to give a total niche biomass prediction, which was then used in the allocation procedure.

Ranking ports by likelihood of marine NIS entry

We calculated an entry likelihood score from ballast discharge and biofouling propagule pressure on a scale of 0 (lowest) to 1 (highest). That is, the mean values from the estimated prediction distribution were scaled to each of the respective discharge and biofouling amounts by the maximum amount within the ports, which sets the scaled score to 1 for the port with the highest amount. We did not subtract the smallest so that no port was allocated a weight of 0. These scores were then averaged to give the relative entry likelihood score for each port. Weights were then calculated in proportion to these relative scores. The weights sum to 1 across the 15 ports and could, therefore, be used to allocate total surveillance effort among ports relative to the likelihood of entry of marine NIS for each port.

Results

Ballast discharge

We fitted the KNN model using all combinations of vessel type and arrival port as categorical variables, DWT or date of arrival as the nearest neighbour matrix, and k = 1,3,5nearest neighbours to the model data set. Results of the cross-validation in Table 5 (Suppl. material 1 – part 7) showed that the KNN that grouped by both port of arrival and vessel type included DWT for calculating nearest neighbours, and using five nearest neighbours performed the best, i.e., this combination of distance variables had the smallest RMSE value (88732.8 for k = 5). A dataset of years 2006–2007 was used to test KNN validation by comparing the mean of predicted ballast water values (as predicted by the bootstrap) with the true values (Suppl. material 1: Fig. S18 in Suppl. material 1 – part 8). Based on the results of the KNN validation summarised in this Fig., the KNN models performed reasonably well for prediction; the observations follow the 1:1 line and double standard deviation lines for the port-level discharge mostly cross the line.

Predicting contemporary ballast discharge

The best model arising from the cross-validation fitting study of the KNN algorithm as selected was used to predict contemporary ballast discharge per port. For this application, the full historical data (1999–2007) were used for training. Fig. 1 shows the mean and 10% and 90% quantiles as estimated by bootstrapping. Tauranga, with an average of 2255.73 \pm



Grouping 🕴 2015 🕴 2016 🕴 2017 🕴 Averaged

Figure 1. Predicted ballast water discharge (kilotonnes) per port for the contemporary data. Data are the mean and standard deviation of total volume as estimated by bootstrapping. The figure displays the mean and 10% and 90% quantiles of the bootstrap distribution.

292.86 kilotons, had the highest predicted average ballast discharge across the years 2015-2017, followed by New Plymouth (1025.22 \pm 231.89), Lyttelton (907.44 \pm 129.39), Taharoa (831.84 \pm 242.45), Napier (812.54 \pm 191.47), Whangarei (624.92 \pm 165.55), and Gisborne (575.33 \pm 160.74). For a port receiving a high number of vessels, Auckland had a low volume of discharge by comparison. Following an increase in 2016, the predicted ballast discharge decreased in 2017 in almost all ports (Fig. 1). The lowest predicted values for ballast discharge were observed in Milford Sound, Picton and Bluff, and zero for Westport.

Biofouling

Based on the Random Forest statistics, e.g., Gini impurity index and MSE (mean squared error), important variables for biofouling prediction were days since last antifouling paint, niche area, vessel speed and DWT. Variability in the modelling was accounted for by predicting quantiles of the vessel biomass (Fig. 2).

As summarised in Table 1, Auckland had the highest average predicted biofouling exposure, followed by Tauranga, Lyttelton, Napier, and Wellington. Ports with very low biofouling exposure were Whangarei, Gisborne, Picton, and Taharoa. Fig. 3 shows the variation of the actual biofouling and the prediction distribution, which demonstrates that the model is not of particularly good quality. That is, some of the data points, particularly for higher values of biofouling mass, are well below the 1:1 line, indicating an underestimation of higher values, which reflects regression to the mean.



Figure 2. Predicted vessel biofouling exposure (tonnes) to each arrival port for the contemporary data (2015, 2016, 2017 and combined years). The median (dot) and 10% and 90% quantiles (upper and lower lines) of the prediction distribution are displayed.

Table 1. Predicted biofouling exposure and 10–90% quantile for the ports with the highest average biofouling mass as illustrated in Fig. 2.

Port	Predicted biofouling exposure (tonnes/year)	10–90% quantile (tonnes/year)
Auckland	70	58.4 - 86.02
Tauranga	65.37	49.62 - 79.82
Lyttelton	30.83	23.11 - 39.1
Napier	26.48	18.81 – 36.22
Wellington	25.81	15.44 - 37.48

Ballast water and biofouling exposure per port

Finally, for each arrival port, the biofouling exposure can be compared with the ballast discharge (Fig. 4). This shows that the arrival ports of Auckland and Tauranga have the highest exposure for biofouling, and Tauranga has the highest volume of ballast discharge (i.e., highest entry likelihoods). The next cluster of ports comprises New Plymouth, which had the second highest ballast discharge but received much lower biofouling exposure than many other ports; Wellington, which had low volume of ballast discharge and high exposure to biofouling; and Lyttelton and Napier, which seemed to have relatively high levels of exposure to both ballast discharge and biofouling. The balance of ports have relatively low exposure.

Based on the relative likelihood of entry of marine NIS via these two pathways, the highest surveillance weights would be allocated to the ports Tauranga and



Figure 3. The variation of the actual biofouling mass (g/m2) versus the predicted biofouling mass (g/m2). The vertical grey lines represent the 10% and 90% quantiles of predicted values. The diagonal line shows the 1:1 relationship.



Figure 4. Predicted vessel ballast water discharge (kilotonnes) vs biofouling exposure (tonnes) per port. The figure displays the mean and 10% and 90% quantiles of the bootstrap distribution for ballast water and from the prediction distribution for biofouling. The values are the average of ballast water and biofouling mass over years 2015 – 2017.

Auckland, followed by Lyttelton, Napier, New Plymouth, and Wellington, whereas the lowest surveillance effort would be allocated to the ports Gisborne, Timaru, Bluff, and Picton.

Priority ports for marine surveillance

The relative entry likelihood weighting, calculated from the mean values of the estimated prediction distributions and scaled for the respective ballast discharge and biofouling exposure, is provided in Table 2. Based on these results, the ports with

Port Total ballast discharge Total biofouling exposure Relative Relative allocation	
relative surveillance effort allocation weights.	
Table 2. Relative entry likelihood for each port for ballast water discharge and biofouling exposure, a	and

Port	Total ballast discharge	Total biofouling exposure	Relative	Relative allocation
	(kilotonnes)	(tonnes)	entry	weight
			likelihood	
Tauranga	2,205,593	64.73	1	0.23
Auckland	307,807	69.53	0.59	0.13
Lyttelton	904,603	30.07	0.43	0.1
Napier	823,096	26.19	0.38	0.09
New Plymouth	1,042,600	9.80	0.31	0.07
Wellington	288,243	25.49	0.25	0.06
Nelson	534,271	16.38	0.24	0.06
Dunedin	530,440	14.45	0.23	0.05
Whangarei	609,168	8.51	0.2	0.05
Taharoa	842,739	0.54	0.2	0.05
Gisborne	581,713	4.03	0.16	0.04
Timaru	247,965	12.82	0.15	0.04
Bluff	213,933	9.05	0.11	0.03
Picton	208,567	2.76	0.06	0.02

higher likelihood of marine NIS entry were Tauranga, Auckland, Lyttelton, Napier, and New Plymouth with scores of 1, 0.59, 0.43, 0.38, and 0.31, respectively. Tauranga was assigned a relative allocation weight of 0.23, followed by Auckland (0.13), Lyttelton (0.1), and Napier (0.09). New Plymouth, Wellington, Nelson, Dunedin, Whangarei, and Taharoa were ranked next based on their surveillance allocation weights. Gisborne, Timaru, Bluff, and Picton had the lowest relative weighted scores. There was a positive relationship between the number of vessel arrivals per port and the entry likelihood scores ($r^2 = 0.75$). Ports where the entry likelihood is greater than might be predicted by numbers of arrivals alone include Tauranga, Napier, New Plymouth and Taharoa, where there are relatively large proportions of bulk carriers or tankers. Ports where entry likelihood seems lower include Auckland and Wellington. To investigate the variability of the ranking of sites, this allocation was also performed to each of the bootstrap replicates. Fig. 5 shows the histogram of ranks from this procedure.



Figure 5. Rank histograms for surveillance allocation, as estimated by bootstrap and prediction simulation.

Discussion

We developed a systematic methodology that can be used to adapt surveillance effort over time in response to changes in propagule pressure. The methods can be applied to port systems in other countries or to other invasion pathways. Summarised at the highest level, the study applied model-based estimation to activity data, where the activity data were contemporary transits of marine vessels. In the case of ballast discharge, the model-based estimation was non-parametric, using KNN to identify the vessel transit trajectories that were most similar to the contemporary records on the available subset of fields. In a sense, the contemporary transit data reweighted the historical ballast discharge records, and the significant changes in activity observed in Tauranga were reflected in its enhanced likelihood of marine NIS entry via this pathway. For the biofouling exposure, we predicted vessel-level biofouling using random forests, and its uncertainty using quantile random forests, and computed port exposure directly from the contemporary transit records. Key in each case was the availability of contemporary activity data that could be used to calibrate the relative likelihood of marine NIS entry experienced by each port. In this system as in many others, the likelihood of entry scales smoothly with the exposure.

Based on this study, Tauranga had the highest predicted joint ballast discharge volume and exposure to biofouling. This is despite Auckland receiving the highest number of vessel visits in almost all years (Suppl. material 1: Fig. S5). It has previously been shown that total number of visits alone is not a reliable predictor of propagule pressure (Verling et al. 2005), and our results reinforce this outcome. Although the busiest ports – such as Auckland, Tauranga, and Lyttelton – had the highest relative exposure to ballast discharge and biofouling, other highly visited ports such as Wellington and Nelson had lower levels when compared to less visited ports such as Napier and New Plymouth. Several studies have relied on vessel visits as a proxy for propagule pressure in ballast water (Drake and Lodge 2004) and this might be a source of error due to significant differences between vessel types and their characterises and operational profiles (Minton et al. 2005; Verling et al. 2005). For example, Carney et al. (2017) reported that the increase in annual ballast discharge was not because of an increase in total vessel arrival numbers, but instead resulted from an increase in bulk carrier traffic. Thus, it seems that considering vessel type, size and discharge capacity will provide a better predicator of ballast discharge volume for the vessel.

Higher entry likelihood at Tauranga is likely related to this port having the highest number of discharge declarations, vessel visits and DWT. Tauranga was also visited with the highest number of bulk vessels, which along with containers had the highest discharge counts in the historical data. Considering that bulk vessels had the highest DWT and discharge volume among the vessel types, it is not surprising that their size and discharge volume contributed to their higher likelihood of marine NIS entry in Tauranga. Increase in ballast discharge in Tauranga, which reached its peak in 2016, was accompanied by the highest number of bulk carriers and containers visiting this port in 2016. Predicted ballast discharges were higher in 2016 mainly because the total annual number of vessels visiting New Zealand ports was highest in 2016; all the vessel types except for general cargo visited New Zealand's ports more in 2016 and had a higher DWT in this year. The total number of visits decreased in 2017, along with the numbers of visits by almost all vessel types including those with high DWT. This can explain the decrease in ballast water predicted values in 2017.

In several studies, ballast discharge volume and frequency have been reported to vary significantly by ship type (Verling et al. 2005; Minton et al. 2015), with the largest volumes of ballast water from bulkers and tankers (McGee et al. 2006; Cordell et al. 2009; Carney et al. 2017). Vessel type was consistently suggested as a reliable predictor of biofouling, as different vessels vary in the size and number of niche areas (Lacoursière-Roussel et al. 2012; Lane et al. 2018). Based on the results of cross-validation and model fit statistics, vessel type was not included as a variable in our models, but the variation may have been more efficiently represented by the included predictors, e.g., DWT. In this study, niche area and days since antifouling mass. Time since last cleaning and last antifouling paint also played a role in the increased likelihood of entry of marine NIS from biofouling (Floerl and Inglis 2005; Drake and Lodge 2007; Lacoursière-Roussel et al. 2012). Biofouling mass on the vessel might also vary depending on the other factors such as vessel speed as lower speeds and inactivity appear to give the fouling communities opportunity to settle (Minchin and Gollasch 2003).

As noted earlier, unregulated ballast discharge is an important pathway for marine NIS spread. Consequently, information about ballast discharge quantities, along with detailed information on ballast discharge mitigation measures such as open-ocean ex-

change, would be valuable for risk analysis. This information would enable reporting of potentially risky discharge events, which could then be used to inform the ports that were most in need of surveillance. However, such records were not available for the contemporary data, and only intention-to-discharge records were available in the historical data.

This study predicted the amount of discharged ballast water and biofouling mass per port at the voyage level, and the ports where the discharge occurred. The predicted values of discharged ballast water and biofouling mass were used to review New Zealand's marine biosecurity surveillance programme by estimating the relative likelihood of entry of marine NIS at each port and allocating surveillance effort between ports accordingly. Some studies have aimed to identify hot spots of marine NIS exchange via ballast water (Drake and Lodge, 2004), or possible source ports and countries for the arrival of a specific NIS by analysing international shipping networks and generating pathway simulations to help optimise shipping container inspection protocols (Paini and Yemshanov 2012). This study differs from previous studies in that a combination of information about vessel traffic patterns and vessel characteristics was used to identify ports likely to receive marine NIS, whilst including a measure of propagule pressure associated with each vessel arrival. Other studies tried to prioritise locations for tracking biological invasions, but used less exhaustive analyses and were more descriptive to illustrate the utility of the database (e.g., Molnar et al. 2008) or to prioritise the pathways that pose the greatest threat (e.g., McGee et al. 2006). In comparison, we developed a systematic methodology that can be used to adapt surveillance effort over time in response to changing vessel profiles and can be applied to ports in other countries or globally. The techniques used in this study allowed us to consider multiple discharges during a voyage for ballast water analysis, and to account for biofouling presence and mass while predicting voyage-level, port-level, and annual propagule pressure.

This study has limitations. We did not incorporate other factors that might mitigate or exacerbate entry likelihood, such as the efficiency of ballast water exchange or treatment in removing marine NIS (Minton et al. 2005), or the influence of transit time on ballast water organism survival prior to discharge (Cordell et al. 2009). Our analysis gave equal weighting to the importance of ballast water and biofouling as pathways for the introduction of marine NIS. Although numerous studies have attempted to summarise the relative importance of these and other pathways for the introduction of marine NIS based on records of established species, such studies typically include many historical (e.g., pre-1950) introduction records, hence the numbers of marine NIS attributed to each pathway may not reflect contemporary patterns of transport. Moreover, many marine NIS can be transported within ballast water and as biofouling, making it difficult to distinguish the relative contribution of each pathway (Fofonoff et al. 2003).

Drake and Lodge (2007) estimated the abundance of fouling organisms on a typical merchant vessel to be between 5.85×10^5 and 6.19×10^6 individuals, which is within the range of abundance of organisms contained in untreated ballast discharges ($2.63 \times 10^5 - 9.83 \times 10^6$ individuals; Minton et al. (2005)). While these gross metrics of per vessel propagule pressure are comparable, it is unclear whether

organisms are equally likely to be introduced, or if introductions by each pathway have the same likelihood of survival and establishment. In the absence of this information the standardised pathway weightings seem appropriate. In aligning surveillance with contemporary patterns of international shipping it is also important to consider other influences on the allocation of survey effort. For example, our analysis did not include port arrivals by small ("recreational") craft, which also carry burdens of biofouling (Floerl et al. 2008; Lane et al. 2018). More than 600 small marine craft enter New Zealand waters each year, with around two thirds of these clearing Customs in Opua. Other significant places of first arrival for small craft are Auckland, Whangarei and Picton which, with Opua, account for more than 90% of annual arrivals. We also did not consider the relationship between surveillance effort and detection probability at the scale of the port, which can be influenced by the size and dispersion of habitats suitable for establishment by marine NIS. For example, the total area of Waitematā Harbour, Auckland, is at least 3 × that of Nelson Harbour and contains the Port of Auckland and 11 marinas for small craft which are distributed from the mouth to the head of the harbour (Morrisey et al. 2012). Gaining equitable survey sensitivity across the surveyed ports requires a proportionately larger sample effort in larger harbours.

Specific policy outcomes regarding surveillance are beyond the scope of this paper. Nonetheless, the change in the pattern of vessel transits between the early 2000's and the contemporary data show that the propagule pressure has sharply increased in Tauranga relative to the other ports, and is now similar to that of Auckland. Further, Napier is subject to similar levels of propagule pressure to locations currently included in the MHRSS programme.

Conclusions

We developed a systematic methodology that can be used to adapt surveillance effort over time in response to changing vessel arrival patterns and types, and that can be applied to maritime ports in other countries or to other invasion pathways. Aligning survey effort with marine NIS entry likelihoods will increase the likelihood of early detection and improve management outcomes. The systematic likelihood-based methodology designed here is flexible and can be applied to surveillance programmes at any time if changes to propagule pressure occur.

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Supplementary material I

Supplementary materials

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Data type: Docx file.

Explanation note: This is a file that contains the supplementary analyses.

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