The stone pine, *Pinus pinea* L., a new highly rewarding host for the invasive *Leptoglossus occidentalis*

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Academic editor: Matt Hill | Received 12 October 2018 | Accepted 30 November 2018 | Published 21 December 2018


**Abstract**

The invasive seed bug *Leptoglossus occidentalis*, a species native to Western North America, is of major concern for the producers of stone pine seeds in the Mediterranean countries. The large size of these edible seeds and their nutritive content may represent a pull factor for the seed bug. Cone and seed traits of three main Mediterranean pine species: *P. pinea*, *P. pinaster*, and *P. halepensis*, were evaluated. Preference trials with cone-bearing branches, individual cones and seeds were conducted to test host preference among the three host species.

Considering the kernel size, stone pine seeds provide 4 to 13 times more reward than *P. pinaster* and *P. halepensis* seeds, respectively, but also needed a greater effort to be reached as measured by coat thickness. Still, the benefit/cost ratio was higher on *P. pinea*. Individual seeds and cones of *P. pinea* were 2 to 3 times more consumed than those of the two other pine species. However, branch preference trials did not reveal any difference in bug visits. Moreover, adults manifested strong group behaviour on branches, frequently dissociating into two persisting groups. The implications of these results for *P. pinea* producing areas are discussed.

**Keywords**

seed feeder, host preference, *P. pinea*, *P. pinaster*, *P. halepensis*


Introduction

The invasive seed bug, *Leptoglossus occidentalis*, Heidemann 1910 (Hemiptera: Coreidae), originating from Western North America, was first recorded in Europe in Italy in 1999 (Taylor et al. 2001). Due to its high dispersal ability, and also probably assisted by more than one introduction event, this bug quickly spread all over Southern and Central European countries and later to the whole continent (Lesieur et al. 2018). Besides of Europe, there has also been new records on other continents in the last decade: Asia (Ishikawa and Kikuhara 2009, Zhu 2010, Ahn et al. 2013), Africa (Jamâa et al. 2013, Gapon 2015) and more recently, South America (Faúndez and Rocca 2017).

With the European invasion, this polyphagous insect, which feeds on cones and seeds of many conifer species in its native range (Koerber, 1963), encountered new potential hosts. In Europe, it has been observed feeding on Mountain pine (*Pinus mugo*), European black pine (*P. nigra*), Scots pine (*P. sylvestris*), Maritime pine (*P. pinaster*), Aleppo pine (*P. halepensis*) and on Stone pine (*P. pinea*) (Villa et al. 2001, Tescari 2004, Tiberi 2007, Kment and Baňař 2008, Lis et al. 2008, Rabitsch 2008, Roversi 2009, Petrakis 2011, Hizal 2012). The last three hosts are the predominant pines species in the Mediterranean region of Europe. Maritime pine is native to the Western Mediterranean Basin, but it can be found in other parts of southern Europe and North Africa (EUFORGEN 2009). The aleppo pine is distributed mainly along the coastal areas in the western Mediterranean regions, being an important forest species in North Africa, France and Italy (EUFORGEN 2009). Finally, the European distribution of *P. pinea* extends across the entire Mediterranean basin, from Portugal to Syria (EUFORGEN 2009). For more than a century, all three have been important species in reforestation and afforestation programs since they can grow in arid and unstable soils (EUFORGEN 2009). Maritime pine is also planted for timber and to extract resin. Aleppo pine, in turn, is also used in the pulp and paper industry (EUFORGEN 2009). Differently, the stone pine *P. pinea* is largely known by its ecological and landscape value but also by its edible seeds, known as Mediterranean pine nuts, of high commercial value, which may reach 100 € per kg in retail (Mutke et al. 2012). In response to this high market value, the plantation area of *P. pinea* has increased in the last decades in several Mediterranean countries, like Spain, Portugal, Italy and Turkey (Mutke and Calama 2016). The high value of this non-wood forest product is precisely the focus of the vast majority of impact studies on the seed feeder *L. occidentalis*, in the Mediterranean countries (Bracalini et al. 2015, Calama et al. 2016, 2017, Farinha et al. 2018a, 2018b). During the last ten years, several countries have reported a strong decrease in cone production and cone yield (i.e., the percentage of commercial kernels per kg of fresh cones) (Mutke et al. 2014), and *L. occidentalis* has been suggested as the most plausible cause of such decrease (Roversi 2009, Bracalini et al. 2013, Mutke and Calama 2016, Parlak 2017). Like all hemipterans, *L. occidentalis* has sucking mouthparts and, it feeds by inserting its stylets deep into the cone until reaching the seed from which it removes the endosperm (Koerber 1963).

Although feeding on a wide range of conifers, *L. occidentalis* seems capable of distinguishing between clones of *P. contorta* (Blatt and Borden 1996, 1999, Richardson
et al. 2017) and between cones of *P. pinea* of different physiological status (Farinha et al. 2018b). This host selection capability strongly indicates that the bug can probably discriminate between different host conifers.

Evaluating host preference of this seed feeder under natural conditions is not a straightforward task due to the difficulty in detecting the bug and the absence of visible damage on cone surface. The only study on *L. occidentalis* feeding preference was carried out under laboratory conditions using individual mature seeds, extracted from cones (Lesieur et al. 2014). Having offered such seeds to adults in choice tests, Lesieur et al. (2014) did not find any preference among several European conifers (*P. sylvestris*, *P. nigra*, *Abies nordmanniana* subsp. *bormuelleriana*, *Larix decidua* and *Picea abies*) compared to the Nearctic Douglas-fir, *Pseudotsuga menziesii* var. *glauca*. However, although quite extensive this study did not include *P. pinea* seeds and to our knowledge, no other studies tested bug preferences for this host species.

Seeds of *P. pinea* are large-sized and highly nutritive which can be a pull factor to a seed-eater like *L. occidentalis*. On the other hand, larger seeds also mean a thicker seed coat which may represent an obstacle for the piercing mouthparts of the bug. We hypothesized that in preference trials using seeds, the bigger individual seeds of *P. pinea* might visually lead to a host preference towards a higher reward whereas the harder seed coat may constitute an additional cost. Seed volume is a proxy to the seed reward and thickness may represent a proxy to the effort.

Selective behaviour in the field is known to operate at sequential levels. First, individuals select a tree, then a cone and lastly a seed to feed upon (Blatt 1997). So, we presumed that host preference may differ regarding the plant component tested; either using seeds, cones, branches or trees. Furthermore, it would be relevant to compare results and discuss the pros and cons when using different methodologies.

In this study, our objectives were to evaluate the host preferences of *L. occidentalis* for branches, cones, and seeds of the three main pine species in the Mediterranean Basin, *P. pinaster*, *P. halepensis* and *P. pinea*. For this purpose, we compared cone volume, seed weight and volume and seed thickness of the three species, and then tested bug preferences in choice tests.

**Methods**

Three separated choice experiments using different substrates, cone-bearing branches, fresh last year cones and mature seeds, were conducted to evaluate the preference of *L. occidentalis* adults among *Pinus pinea*, *P. pinaster* and *P. halepensis*. All the experiments were carried out under laboratory conditions. Only adults of *L. occidentalis* were used since nymphs are apterous and thus not capable of actively choosing the tree or the host where they will feed in natural conditions. All individuals came from a permanent laboratory colony with adults collected in Santarém region, Portugal during the summer of 2015. The colony was supplemented each summer with more adults from the same region to avoid consanguinity thus consisting of individuals with mixed ages.
The colony was reared at Centro de Estudos Florestais, Lisbon, Portugal in a climatic room under the following controlled conditions: 21 °C with 60% RH and 14:10 light/dark cycle. Branches and cones from *P. pinea* were used as food source. Trials began by removing experimental adults from the permanent colony and marking them with an individually coloured and numbered label painted in the thorax. All marked adults were put in a cage with cone-bearing branches and seeds of the three hosts during one week. Individuals were then subjected to a 24-hour starvation period after which the trials began. Adults used to replace dead ones were removed from the permanent colony, marked but were immediately placed in the cages or test boxes.

Cone-bearing branches used in trials were collected in different locations for each of the pine species: stone pine branches were collected in Monsanto, Lisboa (38°43.09′N; 09°12.41′W) in a natural pure stand of adult trees; maritime pine branches were collected in Sobreda, Almada (38°38.06′N; 09°12.66′W) in an urban patchy stand; finally, branches of aleppo pine were collected in the university campus, Lisbon (38°72.80′N; 09°12.66′W). Cones / seeds used in preference trials were from branches / cones from the same locations as above with the exception of stone pine seeds which came from a pure, grafted stand in Santarém region, Portugal (39°6.50′N; 08°21.91′W) and maritime pine seeds in the two-choice trail which came from Setúbal region, Portugal (38°34.82′N; 09°11.09′W).

Assessing the differences in size of cones and seeds among the three Mediterranean pine species

The volume of a sample of the cones used in the experiments was measured by displacement of water in a graduated cylinder (n=6 for *P. pinea* and *P. pinaster* and n=12 for *P. halepensis*).

Respecting mature seeds, all that were used in preference trials were weighted at the beginning and at the end of the experiments. At the end of the trials, all seeds from the three pine species were opened, and the volume of the kernels showing no feeding damage were measured by displacement of water in a graduated cylinder with a sensitivity of 0.25 ml. Due to the very small size of *P. pinaster* and *P. halepensis*, these seeds were measured in batches of 20 seeds and then the individual volume was extrapolated. The thickness of the seed coat for each host species was measured on the images collected by the Scanning Electron Microscope (SEM) using Image J software. The coats of three seeds per host were photographed in SEM with 20 measures taken in each photo.

Branch preference trials

Choice experiments were conducted in large cages (100×50×40) cm made up of a wooden frame and mesh walls. Preference among the three host species, *P. pinea*, *P. pinaster* and *P. halepensis* was tested in pairs by offering two potted branches of differ-
ent plant species per cage to ten adults. All branches used in the trials bear cones at all development stages (1st and 2nd year for *P. pinaster* and *P. halepensis* and 1st, 2nd and 3rd year for *P. pinea*). The number of last-year cones (2nd year for *P. pinaster* and *P. halepensis* and 3rd year for *P. pinea*) in the tested branches was the same for the pair *P. pinaster* - *P. pinea* (ranging from 1 to 2 cones each), but not for the pairs *P. pinaster* - *P. halepensis* and *P. pinea* - *P. halepensis* in which the number of *P. halepensis* cones varied between 2 and 5 due to their smaller size. Young conelets (1st cones for *P. pinaster* and *P. halepensis* and 1st and 2nd cones for *P. pinea*) varied in number in all three species between 1 and 4. The experiment was replicated three times, on 21–22 April, 28–29 April and 5–6 May 2016. Branches for each experiment were collected at the end of the afternoon of the day before the start of the experiment and kept in the refrigerator until then. Ten adult bugs were assigned to each of the three big cages. Cage number 1 had three males and 7 females and cages number 2 and 3 had four males and 6 females. All adults were individually marked in the thorax with a colour and number. The group of insects of each cage remained constant in all three trials varying only the host pairs to be tested. Between trials all individuals were kept together in a single big cage in the laboratory under controlled conditions and with branches from all the three hosts. For each trial, the ten adults were introduced into the cages by placing them one by one, within a two minutes interval, at the centre of the cage, between the two potted branches. Individual bug’s behaviour and localization was recorded after that, at 1-h intervals from 8 a.m. until 6 p.m. for two days.

**Cone preference trials**

Three separate laboratory trials, with 3 to 5 days length each, were conducted from April to September 2017, using a video camera BRINNO TLC200 Pro. In each trial, two video cameras each videotaping two plastic boxes (23×20×19) cm simultaneously, were set. Each box contained a small branch of *P. pinea*, a petri dish with wet cotton to keep moisture and small aluminium cases filled with sand to place the cone. In this way, the insect was not allowed to hide underneath the cones. Similarly as in the branch preference trials, the bugs were individually marked with a coloured label. One cone of *P. pinea*, one of *P. pinaster*, two of *P. halepensis* and three adults of *L. occidentalis* were then added to each box. All cones were from last year of development which corresponds to the 3rd year in *P. pinea* and 2nd year in *P. pinaster* and *P. halepensis*. Experiments were carried on in a room under control conditions (26 °C, 60 % RH, 16:8 L:D) from 20–24 April, 2–6 May and 12–14 September of 2017. The videotaping was done using the time lapse function with one picture taken every two minutes, and played back at a speed of one frame per second. Videos were analysed with the program VLC media player 2.2.6 Umbrella for windows (https://www.videolan.org/vlc/index.html). Both the number of times each bug started feeding on a cone (frequencies), and the duration of the feeding was recorded. Feeding was assumed to have occurred whenever stylet insertion was observed.
Testing bug preference for seeds among the three pine species

Two laboratory trials were carried out to evaluate bug preferences for individual seeds of the Mediterranean pines. A two-choice test compared *P. pinea* and *P. pinaster* whereas a three-choice test included the three species. The first trial lasted three weeks whilst the second one lasted four weeks. The experiments were carried out using small plastic boxes (20×15×10) cm with a perforated lid for gas exchange in a climatic chamber under controlled conditions (21 °C, 60 % RH, 16:8 L:D).

The two-choice trials were carried out at INRA Orléans, France, and the three-choice one at Centro de Estudos Florestais Lisbon, Portugal.

In the two-choice experiments boxes containing both *P. pinea* and *P. pinaster* seeds (n<sub>seeds</sub> =5 and n<sub>seeds</sub> =12, respectively) were set (n<sub>box</sub> =2). No-choice, control experiments were conducted using boxes with only *P. pinea* seeds (n<sub>seeds</sub> =10 per box; n<sub>box</sub> =6) and boxes only with *P. pinaster* seeds (n<sub>seeds</sub> =24 per box; n<sub>box</sub> =2). The experiments were carried out in February and March of 2015 with four adults per box.

The three-choice experiment was performed during January and February of 2017. Twelve boxes, each with ten seeds per pine species, acted as replicates. Each box had three adults.

All boxes included for water supply and as a substrate for resting and laying eggs, a twig of *P. nigra* in the two-choice and of *P. pinea* in the three-choice trial. Previous trials using boxes have shown that insect mortality increases greatly when there is no fresh twig inside (personal observation). The use of different pine species was dependent on conifer availability near the laboratory where the experiments took place. In addition to the twig, a petri dish with wet cotton to keep moisture and foam to support the seeds were also added to each box. Control boxes with no bugs were present in both trials. The sex of the adult specimens was not considered since previous studies found no significant differences in the consumption of conifer seeds between sexes (Bates et al. 2000, 2002, Lesieur et al. 2014). Bug mortality was checked every working day, and dead individuals were replaced immediately, using the stock available from the permanent colony. All seeds from the two-choice trial were radiographed before the trial using the HP Faxitron-43855 X-raying apparatus and X-ray sensitive films (Kodak 'Industrex M'), following the procedures described in Roques and Skrzypczyńska (2003) but optimized for the seeds of the pine species tested. Seeds from the three-choice trial were X-rayed at the University of Lisbon, Faculty of Veterinary using the Philips Practix 300 machine and the constants 45Kv / 25mAs<sup>-1</sup>. Only seeds showing no damage on the X-rays were used in the trials. Each seed was followed individually. Kernel consumption was estimated by subtracting the final seed weight from the initial. During the trial, seeds were radiographed every week for four weeks to track the damage by *L. occidentalis*. In the end, all seeds were opened manually, and kernels were separated into damaged and not damaged. Damaged kernels were photographed using a camera Canon 1100 D and their seed coat was carefully analysed under a stereomicroscope and a Scanning Electron Microscope (SEM) TM3030Plus Tabletop microscope Hitachi.
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Statistical analysis

The size of the mature seeds, the thickness of the seed coat and the volume of the cones all had a normal distribution. The analysis was made using a one-way ANOVA, with three levels corresponding to the three-host species (P. pinea, P. pinaster and P. halepensis). Post-hoc pairwise comparisons were made using the Student-Newman-Keuls (SNK) test. In both the cone and branch preference trials we used Generalized Linear Models (GLM) with repeated measures (each bug was an individual with repeated observations). To compare frequencies among cones and branches, we used GLM with a Poisson distribution, log link function, and Wald Chi-Square test. In the branch preference trials, we performed the analysis for the overall data for each pine species combination, pulling the three cages, as well as per cage. Finally, we used GLM with normal distribution and log link function to analyse differences in the feeding duration time in the cone trials. Again, each bug was considered an individual with repeated measures. For the seed preference trials, to compare frequencies among seeds, we used GLM with a Poisson distribution, log link function, and Wald Chi-Square test. In the two-choice seed trial, we compared: i) the mean number of seeds consumed between choice and non-choice tests for each pine species; ii) the mean number of seeds consumed between pine species on both choice and iii) on non-choice tests. In the three-choice trial, we analysed the differences in the mean number of seeds consumed between the three pine species with boxes considered as repeated measures. We further compared the seed weight consumed and the percentage of kernel consumed per host and box in both seed trials using GLM with normal distribution and log link function. Boxes with no consumption were removed from the analysis.

All statistical analyses were performed using SPSS, version 24.0 (IBM Corp., Armonk, New York) with a statistical significance level of 0.05.

Results

Size of cones and seeds and coatw thickness

The volume of last-year cones differed significantly among pine species (F=92.38; df=2; p<0.001), with the volume of P. pinea cones being two and three times larger than those of P. pinaster and P. halepensis, respectively. The weight of mature coated seeds also differed significantly among species (F=10387.92; df=2; p<0.001) as well as the kernel volume (F= 1526.33; df=2; p<0.001), and coat thickness (F= 4681.251; df=2; p<0.001: Table 1). Seed kernel was 4 times larger in P. pinea than in P. pinaster and 13 times larger than in P. halepensis. Seed coat thickness was more than 3 times greater in P. pinea than in P. pinaster and 12 times greater than in P. halepensis. The ratio kernel volume/coat thickness (KV:CT) that could be used as a proxy of benefit/cost for the bug showed a higher value in P. pinea (Table 1).
Branch preference

No host preference was detected in either of the three host pine choice combinations on the frequencies of visits per bug (*P. halepensis* x *P. pinea*: Wald Chi²=2.485, p=0.115; *P. pinaster* x *P. pinea*: Wald Chi²=0.005, p=0.943; *P. halepensis* x *P. pinaster*: Wald Chi²=0.008, p=0.927). Overall, 70% of the individuals remained on the same host species during the trial period (48h) with the few changes happening on the first day. When each cage was consider separately, a significant preference was observed for one branch or the other, whereas preferred host species may differ from one trial to the other for the same host species combination (Figure 1).

As a general trend, we observed that the ten individuals from each cage dissociated into one or two fix groups in the three trials (Figure 1). The record of the specific place in the plant where the adults were revealed that in more than 80% of the observations the insects were resting between the needles or on the last year cones, regardless the host.

Cone preference

We found no differences between the three trials and so results were analysed together. The adults were observed visiting and feeding more frequently on *P. pinea* cones in comparison with cones from the other two species (visiting: Wald Chi²=17.42; p<0.001; feeding: Wald Chi²=15.31; p<0.001). Per feeding meal, the adults also spent more time feeding on *P. pinea* cones in comparison with other cones (Wald Chi²=12.05; p=0.002) (Table 2).

Seed preference trials

**Bug preference between seeds of *P. pinea* and *P. pinaster***

For four weeks, the four individuals always ate two, out of ten, seeds of *P. pinea* per box, either if it was offered alone (non-choice tests) or mixed with *P. pinaster* seeds (choice tests) (Table 3). Conversely, when adults fed on *P. pinaster* alone, the number of seeds consumed was on average 5.5 ± 1.7, which was higher than the *P. pinaster* seeds consumed in choice tests, 0.5 ± 0.5 (Wald Chi²=5.271, df=1, p=0.022). Considering non-choice tests

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**Table 1.** Cone and seed average measures (± SE) of the three host species. Different letters within a column indicate significant differences between the values per host species after ANOVA tests (p-value=0.05) followed by SNK test.

<table>
<thead>
<tr>
<th>Host species</th>
<th>Cone volume (cm³)</th>
<th>Mature seed weight (g)</th>
<th>Kernel volume (KV) (mm³)</th>
<th>Coat thickness (CT) (mm)</th>
<th>KV: CT</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. pinea</em></td>
<td>90.0 ± 7.6b</td>
<td>0.867 ± 0.008b</td>
<td>202 ± 17.0b</td>
<td>1.438 ± 0.030b</td>
<td>140.5</td>
</tr>
<tr>
<td><em>P. pinaster</em></td>
<td>43.0 ± 1.7b</td>
<td>0.062 ± 0.001b</td>
<td>50 ± 11.0b</td>
<td>0.380 ± 0.009b</td>
<td>131.6</td>
</tr>
<tr>
<td><em>P. halepensis</em></td>
<td>27.3 ± 7.7c</td>
<td>0.022 ± 0.000c</td>
<td>15 ± 1.0c</td>
<td>0.117 ± 0.003c</td>
<td>128.2</td>
</tr>
</tbody>
</table>
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**Table 2.** Bug behaviour averages (± SE) in the cone preference trials. Different letters within a column indicate significant differences between the values per host species after GLM tests (p-value=0.05).

<table>
<thead>
<tr>
<th>Host species</th>
<th>Visiting frequency</th>
<th>Feeding frequency</th>
<th>Time spent per feeding meal (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. pinea</em></td>
<td>13.4 ± 4.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.5 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>131.9 ± 25.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>P. pinaster</em></td>
<td>5.1 ± 1.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.0 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>53.2 ± 12.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>P. halepensis</em></td>
<td>3.7 ± 1.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.1 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>62.5 ± 13.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**Figure 1.** Branch preference trials. Bugs distribution per host in each cage and for each host pair comparison in the preference trials using potted branches. Numbers with asterisk on the x axis correspond to male bugs. The absence of bars means that individuals were never observed on the branches during the trial, but remained on the floor or on the walls of the cage.

alone, the number of *P. pinaster* seeds was significantly higher than those of *P. pinea* (Wald Chi<sup>2</sup>=4.74, df=1, p=0.029). Nevertheless, the consumption of seeds expressed on kernel weight consumed was higher on *P. pinea* than *P. pinaster* both on the choice test (Wald Chi<sup>2</sup>=6.800, df=1, p=0.009) and non-choice test (Wald Chi<sup>2</sup>=25.450, df=1, p<0.001).
Table 3. Seed consumption in choice and non-choice trials. Average number (± SE) of consumed seeds per box, kernel weight consumed per box and bug and percentage of the kernel that was consumed in each of the seed preference trials. Different letters within a trial indicate significant differences between the values per host species after GLM tests (p-value=0.05).

<table>
<thead>
<tr>
<th>Type of trial</th>
<th>Host (s)</th>
<th>Seeds (total)</th>
<th>number of seeds consumed</th>
<th>kernel consumed (mg)</th>
<th>kernel consumed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>two-choice</td>
<td><em>P. pinea</em></td>
<td>5</td>
<td>2.0 ± 0.0</td>
<td>11.71 ± 2.53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>97.12 ± 15.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td><em>P. pinaster</em></td>
<td>12</td>
<td>0.5 ± 0.5</td>
<td>1.28 ± 3.58&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.77 ± 21.23&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>non-choice</td>
<td><em>P. pinea</em></td>
<td>10</td>
<td>2.5 ± 0.7</td>
<td>21.03 ± 2.21</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td><em>P. pinaster</em></td>
<td>24</td>
<td>5.5 ± 1.7</td>
<td>4.13 ± 2.12</td>
<td>–</td>
</tr>
<tr>
<td>three-choice</td>
<td><em>P. pinea</em></td>
<td>10</td>
<td>2.7 ± 1.2</td>
<td>23.91 ± 1.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>92.30 ± 10.62&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td><em>P. pinaster</em></td>
<td>10</td>
<td>3.0 ± 1.8</td>
<td>3.69 ± 1.76&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29.14 ± 10.61&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td><em>P. halepensis</em></td>
<td>10</td>
<td>5.3 ± 2.7</td>
<td>4.51 ± 1.55&lt;sup&gt;b&lt;/sup&gt;</td>
<td>39.73 ± 12.26&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Figure 2. Seed three-choice trial. A sample of seeds consumed by *L. occidentalis* on the three-choice trial. Each row corresponds to a different host pine: (from top to bottom) *P. pinea*, *P. pinaster* and *P. halepensis*, with seeds arranged in each row from the less (left) to the more damaged (right). Photographs taken by Canon 1100 D. The marks on the scale correspond to 1mm. (Photos were taken by Charlene Durpoix).

**Testing bug preference among seeds of *P. pinea*, *P. pinaster*, and *P. halepensis***

Seed consumption was observed on 8 out of the 12 boxes (Figure 2). On these boxes overall, there were no significant differences in the number of seeds consumed between species (Wald Chi²=0.011, df=2, p=0.995) (Table 3). The total number of seeds...
consumed was 15, 16 and 15, respectively for *P. pinea*, *P. pinaster*, and *P. halepensis*. However, when considered the weight of the seed kernel eaten by the adults, we found significant differences among pine seeds (Wald Chi²=117.632, df=2, p<0.001). On average the bugs ate more *P. pinea* seed kernel than the two other species (p<0.001) which, in turn, had no difference between them (p=0.726). When analysing the consumption in terms of the percentage of consumed kernel per host in each box and trial differences were obtained in both two and three-choice trial (Wald Chi²=1002.485 df=1, p<0.001, Wald Chi²=15.625, df=2, p<0.001, respectively). The adults consumed a higher percentage of *P. pinea* kernels that the other two hosts (p<0.001 for both comparisons) (Table 3).

**Discussion**

The impact of an invasive species must be assessed at different levels from the individual to the ecosystem processes level (Parker et al. 1999). The seed feeder, *L. occidentalis* is classified as having a negative impact to the native biodiversity at the individual level (herbivory, predation, competition, disease transmission) and, in addition, a negative economic impact (Rabitsch 2008). Indeed, the presence of the bug in the Mediterranean Basin is a severe threat to the Mediterranean pine nut production as very relevant non wood forest product (Roversi 2009, Calama et al. 2016, 2017, Mutke et al. 2017) by causing direct damage to cones and seeds and as putative vector of the fungus *Diplodia pinea* (Luchi et al. 2012, Tamburini et al. 2012). Here we tested the preference of the bug on the three main lowland conifer species in the Mediterranean Basin region, *P. pinea*, *P. pinaster* and *P. halepensis* at three levels of selection: branch, cone, and mature seed. These three pine species significantly differ in the shape of the tree silhouette and also in the cone size, seed size, and seed coat thickness. Cues behind tree selection by cone feeders may be related to these morphology traits (Turgeon et al. 1994), but also to chemical factors which are indicators of the tree nutritional quality or of its level of chemical defence (Schultz 1988). In fact, there are two predominant hypotheses for the process of host selection by insects: first, that an increased abundance of insects is explained by increased plant vigor (Plant Vigor Hypotheses, Price 1991), or secondly, that it is explained by increased plant stress (Plant Stress Hypotheses, White 1969). Studies on how herbivores select the host plant reported responses according to the theory of plant vigor (Moran and Whitham 1988, Waring and Price 1988, Kimberling et al. 1990, Mopper and Whitham 1992, Hull-Sanders and Eubanks 2005, Mitchell 2006), plant stress (Bjorkman et al. 1991, Rappaport and Wood 1994, Cobb et al. 1997, Virtanen and Neuvonen 1999) or even both simultaneously (Fernandes 1992, Pérez-Contreras et al. 2008) depending on the insect species, its trophic sub-guild (e.g. herbivores that feed on growing plant parts seems more likely to attack vigorous plants (Price 1991, White, 1993) or even on the type of experiment (Waring and Cobb 1992), in a review found that on observational studies, White’s theory was prevalent whilst on experimental studies was the vigor hypothesis).
Under natural conditions, *L. occidentalis* has been shown to select a host in a multi-level process. At first, the bug selects a tree, then a cone and finally a seed where to feed upon (Blatt 1997). Regarding the selection of the tree, the bug tends to select vigorous trees, i.e. denser canopies and longer needles, (Farinha et al. 2018b) bearing moderate crops because it is usually a sign of having larger cones and therefore larger seeds (Blatt 1997, Blatt and Borden 1999, Richardson et al. 2017).

With all that has been said in mind, we hypothesized that branch selection by this bug, a polyphagous insect that feeds on growing plant parts, should rely mostly on vigour (e.g., increased resources, higher food quality, and lack of induced defensive compounds) and cone size and not so much on plant species and their chemical profiles.

In our experimental trials using potted branches with cones, the seed bug showed no clear preference between the three host species but rather a preference for one of the two branches on each trial. The plant vigour hypothesis (Price 1991) for the selection of the host may explain this result. The vigour of the chosen branch (e.g. nutritional state, morphology) and the size of the cones on the branch may have been more critical for a polyphagous insect like *L. occidentalis* than the species of the host plant. Although, we choose branches with similar size, with no signs of diseases and always bearing cones of all ages, yet differences on the number of cones and on its physiological status (e.g. nutritional quality, allelochemicals) might have caused differences between host species and between trials. Furthermore, since branches came from different locations and trees, there could be both a tree and site effects.

Preference studies on a related species, *L. phyllopus* (L.), which is also polyphagous, have evidenced that nutritional and host quality issues (wild vs cultivated plants) are more determinant in host plant selection rather than plant species (Mitchell 2006). A study of *L. occidentalis* impact in a *P. pinea* plantation, also revealed higher bug damage on irrigated and fertilized trees than on control ones, showing a bug preference for high vigour trees (denser canopies, greater needles) (Farinha et al. 2018b).

The branch preference trial also revealed an overall trend for *L. occidentalis* to form two groups of individuals per cage, one in each plant. In general, the composition of the two groups remained similar in each cage in all three trials. During the time between trials, all insects (n=30, 10 from each cage) were placed together in a single cage but when they were replaced one by one in the test cage they regrouped in the same way as in the very first trial. Furthermore, once the individuals had chosen one of the plants, they remained there, in 70% of the cases, throughout the experiment. Group dynamics and not an individual host selection is, thus, a more suitable explanation for the results obtained. The gregarious behaviour of this insect has already been proposed by other authors (Koerber 1963, Mitchell 2006).

Preference for a host species was further tested exposing cones to adults in trials using video cameras. The use of video recording can be very informative on the insect preference because it allows capturing the behaviour of the insect continuously. Moreover, the use of cameras with time lapse mode made it possible to process all data since it condenses several days of filming in movies of few minutes. So, by tracking the feeding behaviour, a clear bug preference could be observed for visiting and feeding on
The stone pine, *Pinus pinea* L., a new highly rewarding host for...

*P. pinea* cones. Furthermore, the individuals spent twice more time feeding, by each feeding meal, on this host species than on the other two species. A higher reward per seed would probably keep insects feeding longer times. It has also to be noted that the seed coats were not yet totally hardened inside cones because we used last year cones collected in the spring. Under natural conditions, the larger cones of *P. pinea* could be more attractive since visual stimuli are important to this bug in the process of host selection (Blatt and Borden 1999, Richardson et al. 2017). The higher reward value may further benefit the performance of *L. occidentalis*, when feeding on *P. pinea* seeds, namely through a higher survival rate and faster development, as indicated by Ponce et al. (2017). Bernays and Minkenberg (1997) in an experimental study with seven polyphagous insects (four Lepidoptera and two Hemiptera) came to the conclusion that it is the greater resource availability rather than the nutritional enhancement or differences in allelochemicals among host species that resulted in a higher performance (survivorship, gain in mass and fecundity). We assume that in our cone preference experiments, the larger cones of stone pine represent a greater resource availability comparing to the other hosts.

In respect to seed trials, no clear preference between mature seeds of *P. pinea*, *P. pinaster* and *P. halepensis* was observed if we compare the number of seeds consumed. However, *P. pinea* kernels are much larger than the others. Furthermore, it has been shown that the same insect feeds several times on the same seed and that different insects may also feed on that same seed by sharing the feeding hole (from video recording observations, data not showed) (Farinha et al. 2018a). Therefore, the mass or the percentage of kernel consumed is a better indicator of the real consumption by the individuals. When considering kernel consumption, *P. pinea* emerges as the most consumed host species in all trials. When expressed in percentage of consumed kernel per host species and per box, *L. occidentalis* consumed about 97% and 92% of *P. pinea* kernels in two-choice and three-choice tests, respectively.

Other preference study at the seed level showed that *L. occidentalis* appeared capable of differentiating a viable seed from one infested by chalcid, *Megastigmus spermotrophus* (Hymenoptera: Torymidae), discarding the latter (Blatt 1997). However, since all the seeds used in our trial were first radiographed and only the healthy ones were used, we hypothesize that feeding onset in a given seed might be random or visually determine. After first opening a hole on the seed, feeding activity would be concentrated on that seed, which in the case of *P. pinea* seeds would satiate longer, further requiring less number of consumed seeds per bug. Also, bigger seeds may result in higher nutrients income in shorter periods which optimize the feeding. This result becomes evident when we compare *P. pinea* and *P. pinaster* kernel consumption in non-choice tests (over 5 times more mass consumed in boxes with *P. pinea* seeds) (Table 2). In another preference trial using mature seeds, Lesieur et al. (2014) found no difference between host species. Still, in that study, the size of the seeds did not differ so much among tested host species.

Despite the larger size of *P. pinea* seeds, which constitute a more significant reward, the seed coat implies a higher cost, being three and twelve times thicker than that of
**Conclusions**

In spring or early summer, depending on the climatic conditions, this insect becomes active and begins to search for a site with coniferous where to feed and reproduce. No data can be found on the bug behaviour when leaving its winter shelter except for an inconclusive study by Richardson (2013) in Lodgepole pine seed orchards for two consecutive years. Does it return to the same place as the year before or disperse elsewhere? How does it select the site to colonize? Is there any host preference at this moment?

Among the three main pine species in the Mediterranean Basin, *P. pinea*, *P. pinaster* and *P. halepensis*, our results support evidence that the cones and seeds of *P. pinea* are highly rewarding for *L. occidentalis*. From a nutritional point of view, we may then expect that *P. pinea* trees and plantations may favour *L. occidentalis* population growth. Whereas in seeds and cones there was always a preference trend for *P. pinea*, no clear preference for host species was detected when we used potted branches.

It must be highlighted that the quality of the host plant rather than the species, and the aggregation behaviour of this bug are important factors to take into account when designing the methodology of future host preference studies. Furthermore, larger scale trials are required. The population dynamics of this insect must be a priority research topic. No management plan will succeed without understanding which factors influence the distribution and abundance of this pest, including the availability of, and its performance on, different hosts.

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Global environmental and socio-economic impacts of selected alien grasses as a basis for ranking threats to South Africa

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Academic editor: C. Daehler | Received 14 May 2018 | Accepted 14 November 2018 | Published 21 December 2018

Citation: Nkuna KV, Visser V, Wilson JRU, Kumschick S (2018) Global environmental and socio-economic impacts of selected alien grasses as a basis for ranking threats to South Africa. NeoBiota 41: 19–65. https://doi.org/10.3897/neobiota.41.26599

Abstract

Decisions to allocate management resources should be underpinned by estimates of the impacts of biological invasions that are comparable across species and locations. For the same reason, it is important to assess what type of impacts are likely to occur where, and if such patterns can be generalised. In this paper, we aim to understand factors shaping patterns in the type and magnitude of impacts of a subset of alien grasses. We used the Generic Impact Scoring System (GISS) to review and quantify published impact records of 58 grass species that are alien to South Africa and to at least one other biogeographical realm. Based on the GISS scores, we investigated how impact magnitudes varied across habitats, regions and impact mechanisms using multiple regression. We found impact records for 48 species. Cortaderia selloana had the highest overall impact score, although in contrast to five other species (Glyceria maxima, Nassella trichotoma, Phalaris aquatica, Polypogon monspeliensis, and Sorghum halepense) it did not score the highest possible impact score for any specific impact mechanism. Consistent with other studies, we found that the most frequent environmental impact was through competition with native plant species (with 75% of cases). Socio-economic impacts were recorded more often and tended to be greater in magnitude than environmental impacts, with impacts recorded particularly often on agricultural and animal production (57% and 51% of cases respectively). There was variation across different regions and habitats in impact magnitude, but the differences were not statistically significant. In conclusion, alien grasses present in South Africa have caused a wide range of negative impacts across most habitats and...
regions of the world. Reviewing impacts from around the world has provided important information for the management of alien grasses in South Africa, and, we believe, is an important component of management prioritisation processes in general.

**Keywords**
alien grasses, environmental impact, GISS, impact assessment, impact magnitude, impact mechanism, socio-economic impact.

**Introduction**

Grasses (family Poaceae) are among the most introduced species around the world; they occur on every continent and in various habitat types (Linder et al. 2018, van Kleunen et al. 2015, Visser et al. 2016). Alien grasses are often introduced for their high economic value. They are the source for the most consumed staple foods in the world (cereal grains) (Prescott-Allen and Prescott-Allen 1990), pasturage for livestock in agriculture (Boval and Dixon 2012), energy through biofuels (Pimentel and Patzek 2005), and they are used in alcoholic beverages such as beer and whisky (Solange et al. 2014). Alien grasses have also, however, been introduced to new areas as transport contaminants and stowaways. For example, a study by Whinam et al. (2005) found that the major source of alien grass (such as *Agrostis stolonifera*) introductions into sub-Antarctic islands was the transport used for ship to shore food transfers.

Whether such introductions were accidental or deliberate, and regardless of the many benefits they provide, the introduction of alien grasses can result in invasions that cause substantial negative environmental and socio-economic impacts (Early et al. 2016, D’Antonio and Vitousek 1992, Driscoll et al. 2014). Grasses such as *Andropogon gayanus* have been reported to increase fire frequencies and intensity in fire-prone ecosystems (Rossiter-Rachor et al. 2004, Rossiter-Rachor et al. 2009, Setterfield et al. 2010). Arundo donax is known to change community structure, thereby causing habitat loss for birds and small mammals in the USA (Bell 1997). And in China, *Avena fatua* is reported to cause economic losses of US$500 million annually by invading agricultural land and reducing crop yields (Willenborg et al. 2005).

Less is known about how these impacts vary across different introduced ranges, but it has been suggested that some introduced ranges experience fewer recorded impacts from alien grasses due to context-dependent factors (Hulme et al. 2013); e.g. the level of grass invasions might track variation in fire regimes, or might be an artifact of how well studied invasions are (Visser et al. 2016). Either way, impacts of alien grasses are most likely still increasing due to factors such as climate change and propagule pressure (Chuine et al. 2012, Fensham et al. 2013). We therefore need to understand these impacts and take precautionary measures in order to prevent or reduce them (Hulme 2003, 2006, Keller and Perrings 2011). Impact assessments are cost-effective tools used to estimate the impacts of alien species and help in the decision-making process during the prioritization of limited resources (Jeschke et al. 2014, Kumschick
et al. 2012, Kumschick and Richardson 2013). Impact assessments have also been used to try to identify factors that predict impacts. Studies have found that traits such as a high fecundity, a habitat generalist strategy, a wide native range, a large body size and a large clutch size are associated with high environmental impacts for mammals, birds, and amphibians (Kumschick et al. 2013, Measey et al. 2016), and traits such as height, life form and life history are associated with greater impacts for plant species (Pyšek et al. 2012, Rumlerová et al. 2016). However, traits have generally been much more successful in predicting invasion success than in predicting impact magnitude. Moreover, impact magnitude has been found to be independent of invasion success (Ricciardi and Cohen 2007).

Similar to the ‘invasive elsewhere’ strategy of predicting invasion (Gordon et al. 2010), is the use of records of ‘impact elsewhere’ to quantify the potential impacts of alien species (Kumschick et al. 2015, Ricciardi 2003). This approach can be useful in predicting the impacts of species such as grasses with biased impact records, i.e. uneven research effort across their introduced ranges. This is because it allows species with limited information to be assessed, compared against other species, and be included in management strategies. Furthermore, the approach also facilitates the search for patterns related to the impact mechanisms and magnitudes, which can ultimately lead to a more predictive understanding of invasions.

Here we assess the environmental and socio-economic impacts of selected alien grasses occurring in South Africa by consolidating their impact records across their introduced ranges (e.g. see Kumschick et al. 2015 for examples of this for alien plants and animals in Europe, and Measey et al. 2016 for amphibians). We do this with the aim of providing quantitative estimates in order to determine which alien grasses have the greatest impacts, and to therefore assist decision makers when prioritising which alien grasses to manage. Furthermore, in order to improve our understanding of the likely impacts, we assess which factors contribute to an increased magnitude of impact in alien grasses by investigating habitats impacted by the species across different regions and determining the mechanisms through which impacts occur.

**Methods**

**Species selection**

There are approximately 256 alien grasses introduced into South Africa (Visser et al. 2017). Of these, we assessed impacts for the 58 species that occur as aliens in at least one of the other following regions: Australia, Chile, Europe or the USA. We adopted this approach because: (i) there is a limited number of studies of grass impacts in South Africa; (ii) these regions have a relatively large literature on alien grasses; and (iii) the regions are assumed to be representative of different major biogeographical realms across the world (Visser et al. 2016).
Literature search

We searched for relevant literature on the impacts caused by the selected alien grasses up to June 2016 using the Web of Science, Google Scholar, as well as biological invasion websites and databases such as Centre for Agriculture and Biosciences International (CABI) Invasive Species Compendium (www.cabi.org/isc), Invasive Species Specialist Group (ISSG) Global Invasive Species Database (www.iucngisd.org/gisd), Hawaiian Ecosystems at Risk project (HEAR) (www.hear.org), California Invasive Plant Council Inventory (www.cal-ipc.org). The grass species’ scientific binomial names were used as search terms. We used synonyms and previous species names obtained from the Integrated Taxonomic Information System (ITIS) (www.itis.gov) as search terms for species with no literature record. We then selected relevant publications from the search results based on the titles and abstract content.

We used primary literature when possible, otherwise, we referred to the literature’s reference list to acquire the cited literature, and the full reference to the cited literature was searched in Google Scholar. If we were still unable to access the primary literature, we noted this and recorded the primary literature as it is cited by the secondary source.

A total of 1300 published sources including >100 websites and databases were reviewed; 352 published references and 98 websites and databases were considered for the impact assessment (Appendix 1).

Impact scoring

Different methods have been developed to quantify the environmental and socio-economic impacts of alien species, with recent notable schemes including the Environmental Impact Classification for Alien Taxa (EICAT) (Hawkins et al. 2015) and the Socio-Economic Impact Classification for Alien Taxa (SEICAT) (Bacher et al. 2018). In this study, however, we chose to use the Generic Impact Scoring System (GISS) (Nentwig et al. 2016) (see Hagen and Kumschick 2018 for a comparison of the EICAT, SEICAT, and GISS schemes) as the GISS has been used widely to assess impacts of different species, and we wanted to relate our results with other previous assessments. The GISS classifies impacts into two major classes, namely (1) environmental and (2) socio-economic, with six impact mechanisms assigned for each impact class:

(1.1) impacts on native plants or vegetation through mechanisms other than competition; (1.2) impacts on animals through predation, parasitism, or intoxication; (1.3) impacts on native species through competition; (1.4) impacts through transmission of diseases or parasites to native species; (1.5) impacts through hybridisation; (1.6) impacts on ecosystems (which includes changes in nutrient pools and fluxes, habitat modifications and changes in disturbance regimes); (2.1) impacts on agricultural production; (2.2) animal production; (2.3) forestry production; (2.4) human health; (2.5) human infrastructure and administration; and (2.6) human social life (Nentwig et al. 2016). For each impact mechanism a six-point ranked scale is used, ranging from zero (no impact detectable) to five (highest impact possible at a site) (Kumschick
et al. 2015). The GISS contains definitions and descriptions for the impact mechanisms and the impact scores within them. We assigned an impact mechanism and score to every recorded impact obtained according to the definitions and descriptions of the GISS. Scores can be summed over mechanisms to get a total score per species, with a maximum overall impact score of 60 (12 categories * a maximum impact score of 5 in each category—see details on the scoring system in Kumschick et al. 2015, Nentwig et al. 2016). In this study, we used the maximum impact score recorded per mechanism of each species for both environmental and socio-economic impacts to rank species (see Table 1). This method of aggregating only the maximum impacts per species per mechanism was used by Kumschick et al. (2015); we also adopted it in order to make our results comparable.

Because scores are based on published research, species that receive more research attention might be expected to have higher scores (Pyšek et al. 2008). Therefore, we tested the relationship between the species’ overall impact scores and the number of published papers used per species using a Pearson correlation test (Kumschick et al. 2017). We also tested whether there is a correlation between the species’ overall and maximum impact score in any one impact mechanism using a Kendall’s tau correlation test.

Impacts across habitat types and regions

For each impact reference, we recorded the habitats where the impacts were said to occur, using the habitats classified according to the first level of the International Union for the Conservation of Nature (IUCN) Red List Habitat Classification Scheme (Version 3.1) (www.iucnredlist.org). In cases where the study was not in a natural habitat (e.g. greenhouse or laboratory) or the habitat was not stated, we recorded the habitat as ‘not specified’.

We also noted the country where the impacts occurred for each impact recorded and determined whether the grass species was native or alien in that specific country. Impact records from the native range were excluded from further analyses. We did, however, retain cases where the country was not specified but the grass species was referred to as “alien”, “introduced”, or “non-native”. We assigned each record to one of eight regions based on the location of the country in which the impacts were recorded. We used a Kendall’s tau test to determine the correlation between the maximum impact of alien grasses in South Africa and the maximum impact elsewhere.

Statistical analysis

In contrast to the approach taken above to rank species, when testing the relationship between impact and habitats and region, we used the raw data on impact scores (i.e. each impact record was considered as a separate datum). The impact scores analysed here are therefore ordinal variables in which the scores are ordered (but which closely resemble a logarithmic scale). As such, we used a cumulative link mixed-effects model in the R package ‘ordinal’ (Christensen 2015) to test whether habitats and regions influ-
Table 1. Grasses alien to South Africa and one other region (Chile, Europe, Australia and the USA) ranked according to impacts. The numbers under environmental and socio-economic impacts are the respective sums of the maximum impact scores per impact mechanism of a species. Species that score a maximum of 5 in any one impact mechanism are highlighted in bold. NA indicates no impact found for that species, hence not applicable. Total impact represents the overall sum of the environmental and socio-economic impacts. Species marked with an asterisk* have impacts recorded in South Africa. Literature used and detailed maximum scores per mechanism are available in the Supporting Information (Appendix S1 and Table S1).

<table>
<thead>
<tr>
<th>Species name</th>
<th>Environmental impacts</th>
<th>Socio-economic impacts</th>
<th>Total impact</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortaderia selloana*</td>
<td>7</td>
<td>11</td>
<td>18</td>
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<tr>
<td>Arundo donax*</td>
<td>10</td>
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<tr>
<td>Avena fatua*</td>
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<tr>
<td>Elymus repens*</td>
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<td>Festuca arundinacea</td>
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<td>Nassella trichotoma*</td>
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<td>Sorghum halepense*</td>
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ence impact magnitude. Since we found multiple studies that assess the same impacts for the same species in the same region or habitat, we included species identity, as well as mechanism nested in impact type (environmental or socio-economic) as random factors and impact mechanism, habitat type, and region as fixed effects. We also tested a model in which mechanism nested within impact type was included as a fixed effect but found this made no difference to the results. We did not investigate interactions among predictors because of the limited number of observations. To determine the goodness of fit for the model we calculated pseudo $R^2$ by fitting a null model with no predictor variables and compared it against the full model using the ‘nagelkerke’ function within the R package ‘rcompanion’ (Mangiafico 2016). We tested the significance of fixed effects using analysis of deviance of single-term deletion models tested against the full model using a chi-squared distribution from the ‘drop1’ command. We used least-squares means with $P$ values adjusted using the Tukey method, to determine significant differences between the levels of each predictor (mechanism, habitat and region).

All statistical analyses were performed using R version 3.4.4 (R Core Team, 2018).

### Results

### Grasses ranked by impact

Of the 58 alien grasses selected for impact assessment, we found records of impact for 48 species, i.e. 10 species (Suppl. material 1: Table S1) were data deficient with no record of impact. The species with the highest overall impact score was *Cortaderia selloana* (impact magnitude = 18), followed by *Arundo donax*, *Avena fatua*, *Elymus repens*, and *Festuca arundinacea* (all with impacts of 17, Table 1). However, a different set of species scored the maximum possible impact of five on any one particular impact mechanism, namely, *Glyceria maxima* (animal production), *Nasella trichotoma* (animal production), *Phalaris aquatica* (predation or parasitism or intoxication and animal production), *Polypogon monspeliensis* (animal production), and *Sorghum halepense* (agricultural production) (see Suppl. material 1: Table S1).
We used a total of 352 published literature sources; however, the literature was highly skewed, ranging from one to 23 publications per species. Some literature sources reported on more than one species. We found a significant positive correlation (tau = 0.48, P = 0.006) between the overall impact scores per species and the number of publications used to score the impacts. However, this potentially only affects the relative rankings of species according to impact scores (Table 1), because for the mixed effect model analyses, we did not aggregate maximum records of the species and used each paper as a separate record.

Impact magnitudes across mechanisms

We found that three-quarters (36 out of 48) of alien grass species have records of causing environmental impacts through competition with native species, and half (24 out of 48) of the species have records of causing impacts on ecosystems (Figure 1). We found the fewest records and the lowest overall impact through the ‘plants or vegetation’ mechanism, which according to the GISS includes allelopathy or the release of plant exudates (Nentwig et al. 2016). Most socio-economic impacts are caused through agricultural and animal production, with 29 and 26 cases respectively, while forestry production was represented by few species (Figure 1). The maximum impact possible (5), was recorded for impacts on animals through predation or parasitism, animal production and agricultural production. When comparing scores between impact types, greater impact magnitudes of 4 and 5 were obtained for socio-economic than environmental impacts.

The effects of impact mechanisms, impacted regions, and habitat types on impact magnitude

We found that impact mechanism is the only statistically significant predictor of impact magnitude (P < 0.001, Table 2). Results from the model show that alien grasses have a lower impact magnitude through the transmission of diseases or parasites to native species and greater impacts on native animals through food availability or palatability and intoxication (Figure 2). There is a trend towards greater impact magnitude in Antarctica (Suppl. material 1: Figure S1); however, differences across regions are not significant (P = 0.057, Table 2). We found nine habitats impacted by alien grasses;
Figure 1. Number of alien grass species per impact mechanism for each impact magnitude. On the x-axis are the GISS environmental and socio-economic impact mechanisms, and on the y-axis are the impact scores according to GISS. The size of the points represents the number of species which had the corresponding maximum recorded impact score for that mechanism (out of the 48 species with impact records). See Suppl. material 1: Table S1 for the full details.

Figure 2. The impact magnitude of the 48 studied alien grasses across different impact mechanisms. On the x-axis are the least-squares means of the impact scores as derived from a cumulative link mixed effects model, and on the y-axis are the GISS impact mechanisms with the number of species in brackets. The points represent the impact magnitudes and the error bars represent 95% confidence intervals. Letters on the right side of the confidence intervals are level groupings indicating significant differences among the mechanisms (level groupings with the same letters are not significantly different, comparisons are Tukey adjusted).
However, as with "region" as a predictor of impact magnitude, habitat type was also not a significant predictor (P = 0.49, Table 2), and differences among habitats were not statistically significant (Suppl. material 2: Figure S2). Including mechanism nested within impact type (environmental or socio-economic) as a random effect provided no improvement in model fit (Suppl. material 1: Table S2). However, we kept this nested random effect in the analysis because it accounts and corrects for non-independence of the observations and reflects the actual design of this study.

**Impact of alien grasses in South Africa versus elsewhere**

We found that only 16 of the 58 alien grasses had recorded impacts in South Africa, 13 for inland and three for the offshore islands (Table 1). These impacts were mostly lower than elsewhere, with the exception of *Nassella trichotoma* and *Hordeum murinum* (Figure 3). However, there is no correlation ($\tau = 0.14$, $P = 0.28$) between impacts of alien grasses in South Africa and those recorded elsewhere in the world.

**Discussion**

This study is the first environmental and socio-economic impact assessment to focus specifically on alien grasses. Using the GISS we were able to quantify the impacts of
alien grasses using information from across the globe. This study, therefore, provides a useful overview of the literature on evidence-based impacts of alien grasses and highlights potential risks to South Africa. Furthermore, it shows gaps in the available literature as some species could not be assessed due to a lack of impact studies.

We found that alien grasses generally scored higher for socio-economic than environmental impacts. Grass impact scores were particularly high for agricultural and animal production. This might reflect the large number of agricultural weeds that are grasses (Daehler 1998) or their initial introduction for agricultural purposes (Hancock 2012). Alien grasses scored the lowest for impacts caused via transmission of diseases or parasites to native species, with a maximum score of 2, which represents a minor impact (Nentwig et al. 2016), while the frequency under this mechanism was larger. On the contrary, mechanisms with scarce literature, such as impacts on native animals, obtained higher impact scores. This could be because impacts through the transmission of disease or parasites between plant species are not readily observed in the wild, most of the literature under this mechanism is from small-scale laboratory studies which do not report impacts on the overall population.

Despite most grasses not having very high overall impact scores compared to other species (e.g., Kumschick et al. 2015), many alien grasses scored high across the full range of impact mechanisms (i.e. alien grasses can cause a wide range of environmental and socio-economic impacts) and so had high total impact scores. For example, Cortaderia selloana did not have any individual mechanism score over 3 but has the highest overall score (Table 1) due to the many different mechanisms through which it causes impacts. In contrast, Polypogon monspeliensis and Phalaris aquatica scored the highest impact (5) in certain impact mechanisms, but their overall score is lower. This trend is not observed in other studies, such as the one conducted on alien aquatics by Laverty et al. (2015), where the species with the highest overall score also obtained an impact score of 5 for two different mechanisms. Grasses thus provide an interesting case to explore whether we should be more concerned with invasive species that cause a range of different types of impacts or invasive species that only cause a few types of impacts but with greater magnitude.

Grasses are one of the most cosmopolitan plant families in the world and are present in almost all terrestrial habitats. They also impact a wide range of habitats, as demonstrated in this study. Knowledge about which habitats are most severely impacted by alien grasses is essential for their management. Grasses can cause rapid and dramatic transformation of non-grassy habitats into grass-dominated communities. For example, Bromus rubens and B. madritensis have caused widespread transformation of shrubby systems in the Mojave Desert (DeFalco et al. 2007, Jurand et al. 2013). With regards to regions, we found that Antarctica (sub-Antarctic islands mostly) on average has the highest alien grass impact scores. Grasses such as Agrostis stolonifera reduce moss diversity, liverwort populations, and replace the rosaceous dwarf shrub (Acaena magellanica) with dense grassland patches on Marion Island (Gremmen et al. 1998). It is not clear, however, whether this trend is due to differences in sampling effort or a greater susceptibility of sub-Antarctic islands to impacts than the mainland (Hagen and Kumschick 2018).
However, neither habitat nor region were found to be significant predictors of impact magnitude. This could suggest that the impacts are the same across habitats and regions, but the lack of signal likely also reflects the low sample sizes for most habitat types and some regions. Furthermore, it will be interesting to repeat this study based on a more representative global sample of species (the bias in this current analysis towards grasses alien to South Africa was simply for applied reasons).

When we compare impacts scores of alien grasses with impact scores of studies that assessed other plant taxa (Kumschick et al. 2015, Rumlerová et al. 2016), our results also show that the competition with native plant species is the most frequent mechanism through which alien grasses cause impacts. Four species from our list were previously assessed in those studies (Kumschick et al. 2015, Rumlerová et al. 2016), and our results were similar to them for two of the species (Arundo donax and Paspalum dilatatum), each with a difference of less than 5 between the overall impact scores. However, we obtained higher overall impacts than Kumschick et al. (2015) and Rumlerová et al. (2016) for the other two species (Cortaderia selloana and Hordeum jubatum), each with a difference of 9 and 8 respectively. These differences can be explained by the broader search criteria applied; for example, authors of the above-mentioned studies used keywords such as “invas* or exot* or weed*” in addition to the species name, while we only used the species name as a search term.

Although impacts of alien grasses are poorly studied when compared to other species, such as birds and mammals, we were able to find impact records for more than 80% of the grass species selected for the assessment, which is higher than for other species, such as amphibians (41.3%) (Measey et al. 2016). The average number of papers (5.7) used to score impacts of alien grasses across the globe was also higher than the amphibians and other species (Kumschick et al. 2015, Measey et al. 2016). Similar to the mammals and other plants (Kumschick and Nentwig 2010, Kumschick et al. 2015), alien grasses were also reported to cause impact across all impact mechanisms. This might be because grasses occur across a wide range of sectors and habitats, which allows them to exert impact across all mechanisms. When prioritising management of all alien species, our list can be compared to other assessments conducted for other species, such as birds, amphibians, mammals, and aquatic species (Kumschick and Nentwig 2010, Laverty et al. 2015, Measey et al. 2016, Nentwig et al. 2010). However, it is important to note that impact assessments of some of those species are based on impacts recorded only in Europe and not globally, which may cause a bias to the overall impact scores. More impact studies are still needed for alien grass species, especially when it comes to species with no impact records across all introduced ranges, but with taxonomic characteristics of invaders (such as Bambusa balcooa, Canavan et al. 2016). It will be interesting to see if the findings of Canavan et al. (2018a), that bamboos have similar impacts in their native and alien ranges are the same for other grasses or perhaps only other tall-statured grasses (Canavan et al. 2018b). However, we suspect there are qualitative differences between the impacts in the native and alien ranges, for the grasses studied here, as the impacts observed are not primarily a response to human disturbance.

Two species were scored as causing very high impacts (4 or 5) outside of South Africa, but only low levels of impact (1 or 2) in South Africa. For instance, Glyceria maxima obtained
a score of 5 because it is associated with the death of livestock through poisoning in Australia (Barton et al. 1983), but such impacts have not (yet) been recorded in South Africa. This can flag species that could potentially cause high impacts in South Africa and which should therefore be monitored, or preventative measures put in place to limit such impacts occurring in future. In most other cases the impact elsewhere was either the same or slightly higher than that recorded in South Africa, except for Agrostis stolonifera, Hordeum murinum, and Nassella trichotoma. This included two species (Nassella trichotoma and Hordeum murinum) whose impacts in South Africa were one level higher than elsewhere. For example, Nassella trichotoma obtained a score of 5 in South Africa and 4 elsewhere (in Australia) for impacts on animal production by reducing livestock carrying capacity and pasture production (Klepeis et al. 2009). The lack of correlation between impacts found in South Africa and elsewhere should, however, be assessed with caution – it is indicative of a research gap. Records of impacts are generally fewer in South Africa (with a maximum of five sources per species and an average of 1.9) and even lacking for most species. Alternatively, it could indicate that there is an impact debt (Rouget et al. 2016), i.e. species have not reached their full impact potential in South Africa (yet), as species with more information in South Africa did not show higher similarities in impact magnitudes to elsewhere. Finally, South Africa might be more resilient to grass invasions, and impacts are actually lower here (Visser et al. 2017). These hypotheses warrant more research and can only be disentangled once more data become available.

In summary, the lack of statistically significant differences in impact magnitudes across habitats and regions for alien grasses suggests that impact in this group is not habitat or region specific as in other groups (cf. Hulme et al. 2013, Pyšek et al. 2011). As such, we recommend that different habitats should be equally considered for alien grass impact management. While we recommend that impact scoring schemes, such as the one used in this study, should be incorporated in the decision-making processes for alien species management, we caution that extrapolations from other invaded regions indicate potential and not actual impacts.

Acknowledgements

We acknowledge the financial support from the Department of Environmental Affairs through their funding of the South African National Biodiversity Institute. We also acknowledge the DST-NRF Centre of Excellence for Invasion Biology for their support.

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**Appendix I**

Literature, websites, and databases used to score environmental and socio-economic impacts of 58 alien grass species according to the GISS.


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Global environmental and socio-economic impacts of selected alien grasses...


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385. http://keys.server.lucidcentral.org/weeds/data/030308000b07490a8d040605030c0f01/media/Html/Avena_barbata.
Supplementary material 1

Table S1, Table S2, Figure S1
Authors: Khensani V. Nkuna, Vernon Visser, John R.U. Wilson, Sabrina Kumschick
Data type: species data
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.41.26599.suppl1

Supplementary material 2

Figure S2
Authors: Khensani V. Nkuna, Vernon Visser, John R.U. Wilson, Sabrina Kumschick
Data type: statistical data
Explanations note: The impact magnitude of the 48 studied alien grasses across different habitats. The impact magnitudes on the x-axis are the least-square means of the impact scores as derived from a cumulative link mixed effects model. On the y-axis are the habitat types impacted by alien grasses and in brackets is the number of species with records in that habitat. The points represent the impact magnitudes and the error bars represent 95% confidence intervals. Letters on the right side of the confidence intervals are level groupings indicating no significant differences among the habits. Comparisons are Tukey adjusted.
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Link: https://doi.org/10.3897/neobiota.41.26599.suppl2
Variation in phenology and overall performance traits can help to explain the plant invasion process amongst Mediterranean ecosystems

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Abstract

Plant traits such as phenological development, growth rate, stress tolerance and seeds production may play an important role in the process of acclimatisation to new environments for introduced plants. Experiments that distinguish phenotypic plasticity from ecotypic differentiation would allow an understanding of the role of plant traits in the invasion process. We quantified the variation in phenological and overall performance traits associated with the invasion process for three herbaceous species native to Spain and invasive to Chile (Trifolium glomeratum, Hypochaeris glabra and Leontodon saxatilis). We grew plants from native and exotic populations along rainfall gradients in outdoor common gardens, located in the native and the introduced ranges and measured plant survival, phenology (days to flowering), biomass and seed output. Days to flowering was positively correlated with precipitation of the origin population for T. glomeratum and the native populations of H. glabra, but this pattern was not adaptive, as it was not associated with an increase in performance traits of these species. Phenology may instead reflect ecotypic differentiation to the environmental conditions of the original populations. Comparison between ranges (i.e. performance in both common gardens) was only possible for L. saxatilis. This species showed little...
variation in phenology and both native and exotic populations had higher fitness in the introduced range. This suggests that plasticity enhances invasiveness through increased propagule pressure in the novel environment. Our findings highlight the utility of common garden experiments in examining patterns of phenological and performance traits that relate to species invasiveness.

**Keywords**
Asteraceae, biological invasions, biomass, common garden, *Hypochaeris glabra*, invasiveness, *Leontodon saxatilis*, phenology, precipitation, range expansion, seed output, survival, *Trifolium glomeratum*

**Introduction**

Despite recently gaining attention and considerable resources having been invested into studying habitat invasibility and species invasiveness (Richardson and Pyšek 2006, Guo et al. 2015), understanding the role played by invasive plant traits in the process of acclimatisation to the novel conditions along the introduced range still remains a key knowledge gap in invasion biology (but see MacDougall and Turkington 2005, Molina-Montenegro et al. 2010, Moravcová et al. 2015). Some overall performance traits have been suggested to be crucial for plant invasiveness, such as plant growth rate, environmental tolerance, phenological development and seed production (Noble 1989, Pyšek and Richardson 2007, van Kleunen et al. 2010, Moravcová et al. 2015). In this sense, it has been shown that greater plant growth and seed output account for the invasiveness of many alien plant species (Grotkopp and Rejmánek 2007), where species producing a greater number of seeds increase their propagule pressure and hence, their chances for establishment. However, the role of plant phenology in biological invasion processes and species invasiveness has often been neglected despite considerable differences in phenological development between native and invasive species having already been pointed out (Wolkovich and Cleland 2011, Godoy and Levine 2014).

Plant invasiveness often involves rapid adaptive evolution and/or genetic drift. Thus, invasive plants often undergo phenotypic differentiation to cope with novel environments through a combination of two processes, phenotypic plasticity and ecotypic differentiation (Maron et al. 2004, 2007, Molina-Montenegro et al. 2013). Phenotypic plasticity is the ability of a plant genotype to modify its physiology/morphology in response to environmental conditions and has been indicated as a mechanism that can mediate the establishment and dispersal in the new area (Valladares et al. 2005, 2006, Rejmánek et al. 2005, Richards et al. 2006, Pyšek and Richardson 2007, Pichancourt and van Klinken 2012). However, plasticity is not necessarily adaptive (i.e. does not always improve fitness) and the role that it plays in invasion processes remains still unclear (but see Chambel et al. 2005). Ecotypic differentiation may occur for the invasive plant species in the introduced range through selection of the optimal phenotype that provides local adaptation in different geographic locations, leading to many genotypes adapting to particular environmental conditions and thus allowing increased fitness (Lande 2009, Molina-Montenegro et al. 2013, 2018a, Martín-Forés et al. 2017c, 2018). For example, in more humid environments, plant phenology can
show a delay which *a priori* gives plants more time to invest in biomass production and display more dispersal units (Pérez-Ramos et al. 2010).

It is known that these two processes can occur very quickly for annual Mediterranean species (Cocks et al. 1982, Small and Lefkovich 1986, del Pozo et al. 2000) that have been expanded beyond their initial distribution centre. As a result, some functional traits are expected to be affected and result in enhanced environmental tolerance and/or up-take of resources (Molina-Montenegro et al. 2018b).

Mediterranean-type ecosystems worldwide are considered as biodiversity hotspots and therefore targets for conservation policies (Myers et al. 2000), but despite many conservation efforts, the frequency and intensity of biological invasions in Mediterranean ecosystems is still considerable (Arianoutsou et al. 2013, Martín-Forés et al. 2017a). In this sense, the Mediterranean climate-type region of central Chile constitutes an interesting natural lab for exploring variations in functional traits caused by these mechanisms. Associated with the Spanish conquest that took place in the 16th century, many exotic species were accidentally introduced into Chile (Martín-Forés et al. 2012, 2017a) and became naturalised in the Mediterranean climate region of central Chile.

Previous studies centred in the Mediterranean-type region of central Chile have shown a combination of these mechanisms for some species. For instance, for the invasive Asteraceae *Taraxacum officinale*, both plasticity and ecotypic differentiation for various traits were found in relation to latitudinal (Molina-Montenegro and Naya 2012, Molina-Montenegro et al. 2013, 2018a) or altitudinal (Molina-Montenegro et al. 2012) gradients in Chile. Additionally, ecotypic differentiation along environmental gradients has been observed for phenological development of *Medicago polymorpha* in Chile (del Pozo et al. 2000, 2002a, 2002b). In relation to performance traits, two of the most common invasive species in Chile, the Asteraceae *Leontodon saxatilis* subsp. *rothii* and *Hypochaeris glabra* showed increased propagule pressure and longer distance dispersal for exotic populations and at the introduced range (Martín-Forés et al. 2017c, Martín-Forés et al. 2018).

In particular, this study focuses on three annual species that are native to Spain and invasive to Chile, being broadly distributed in both the native and the introduced ranges, far beyond the Mediterranean climate distribution (Martín-Forés et al. 2012, Casado et al. 2015, 2018; See Suppl. material 1: Figure S1 for detailed information). Here, we bring together and compare plasticity and ecotypic differentiation not only on performance traits but also on phenology of two representatives of the Asteraceae family, *Leontodon saxatilis* subsp. *rothii* and *Hypochaeris glabra* (Martín-Forés et al. 2017, Martín-Forés et al. 2018) and one of the Fabaceae family, *Trifolium glomeratum* that has been selected because of its importance as a fodder plant.

Since the introduction of these three species into Chile (according to the first record, no more than 120 years ago; Castro et al. 2005), they have encountered different abiotic and biotic conditions in the introduced range from those of their native range (i.e. edaphic and climate characteristics, photoperiod, land use patterns, livestock grazing) as well as community interactions such as competence, tolerance and facilitation.
processes (Martín-Forés et al. 2015, 2016, 2017b). Accordingly, studies carried out in both the native and the introduced range of a species have been highlighted as of especial importance because they constitute the most direct test of determinants of invasiveness (Williams et al. 2008, van Kleunen et al. 2010). Thus, the aim of this study was to compare variations in phenology and performance traits associated with the invasion process of *L. saxatilis*, *H. glabra* and *T. glomeratum* into Chile. We used seeds from five Spanish populations and five Chilean populations collected along rainfall gradients in both countries and we evaluated all the populations in two common gardens located in Madrid, Spain and Cauquenes, Chile, that is in the native and introduced range, respectively. We explored *i*) whether the geographic origin of the plant collections (hereafter populations) could explain differences in plant phenology and performance traits within the same common garden trial and *ii*) whether individuals of the three species responded through phenotypic plasticity to the different climatic conditions existing in the two common garden trials regardless their population. The native populations of the three species have been longer exposed to local environmental conditions in the native range than exotic populations in the introduced one; therefore they have had more time to develop local adaptation through ecotypic differentiation. Thus, we would expect Spanish populations to present greater ecotypic differentiation than Chilean ones; if so, the delay in phenology while increasing the amount of precipitation on the origin population would be stronger for Spanish populations. Likewise, if the delay in phenology turns out to be adaptive, plants will display greater biomass and seed output.

**Methods**

**Study area**

The study was conducted in grasslands of the Mediterranean regions of Spain and central Chile (typically called *dehesas* and *espinales*, respectively) used for extensive livestock grazing, especially sheep and cattle. These grasslands present slightly acidic soils and are adapted to Mediterranean-type climate, characterised by having scarce precipitation in summer (drought period from June to September in the Northern hemisphere and from December to February in the Southern hemisphere).

For the three species, we selected five Spanish native populations and five Chilean exotic populations representative of the rainfall gradient existing in the Mediterranean regions of both countries. In Chile, the five populations were located in the central region (from 32°31' to 37°00'S and 70°46' to 72°34'W), with mean annual precipitation ranging from 300 to 1200 mm (Table 1; Suppl. material 2: Figure S2). In Spain, the five populations were located in the centre-west of the Iberian Peninsula (from 38°16' to 39°33'N and from 5°23' to 6°20'W), with mean annual precipitation ranging from 450 to 950 mm (Table 1; Suppl. material 2: Figure S2). The total annual precipitation (mm), mean annual temperature (°C) and number of months with drought period or
water deficit per year, an index calculated as the number of months in which monthly mean temperature is at least double the monthly precipitation, were determined for each population (Table 1). Climate variables were obtained from WorldClim (Hijmans et al. 2005), at a resolution of 30 arc-seconds.

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<td>Ch5</td>
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Selected populations ideally contained the three species studied. Flower heads of *L. saxatilis, H. glabra* and *T. glomeratum* were collected from the five native (i.e. Spanish) and the five exotic (i.e. Chilean) populations in spring of 2010, at the end of flowering periods for most plants (i.e. May-June in Spain and October-November in Chile). Mature flower heads were randomly collected from 50 individuals of each species at each population; the distance between the individuals selected within each population was at least 1 m from each other and they were haphazardly distributed around an area of approximately one hectare (for detailed information about data collection for *L. saxatilis* and *H. glabra*, see Martín-Forés et al. 2017c, 2018, respectively).

Common garden growing conditions

Seeds from the 50 collected flower heads were pooled together. In each range, seeds randomly chosen from each population were germinated in petri dishes on to filter paper and irrigated every two days with 5 ml of distilled water. In the case of *L. saxatilis* and *H. glabra*, peripheral fruits and unbaked fruits were respectively chosen for subsequent planting because of their greater success in pre-germination studies (see Martín-Forés et al. 2017c, 2018 for detailed information). In the case of *T. glomeratum*, seeds were previously scarified by immersing them in boiling water for 5 minutes; afterwards, they were inoculated with *Rhizobium trifolii* before transplanting the seedlings to the common garden to ensure nodulation and nitrogen fixation.
When the radicles of plants (F₂) reached 5 mm, seedlings were transplanted into subplots within two common garden trials, one located at the Faculty of Agronomy of the Polytechnic University of Madrid, Spain (40°26’N, 3°44’W; 600 m a.s.l.; 15 °C mean annual temperature; 484 mm mean annual precipitation) in the native range and the other one located in central Chile, at the Experimental Centre of Cauquenes-INIA, Chile (35°58’S, 72°17’W; 140 m a.s.l.; 14.4 °C; 748 mm mean annual precipitation), in the introduced range. The experiments were set outdoors under semi-controlled conditions where large herbivores were excluded. Planting was conducted directly in the soil when the rain period started, i.e. in June 2012 in Chile and October 2012 in Spain. For each species in the Spanish trial, 20 seedlings of each population were planted in subplots of 200 x 50 cm after removing surface vegetation through ploughing; however, due to space limitations, in the Chilean trial, only ten seedlings of each population were planted and the subplots size was 100 × 50 cm. In both countries, the distance between plants was 20 cm and the separation between neighbouring subplots was 30 cm. A complete randomised design was used with three replicated subplots per population. Thus, there was a total of 87 subplots within each site: 45 containing populations from Spain (three species × five populations × three replicates) and 42 containing populations from Chile (three species × five populations (four in the case of L. saxatilis) × three replicates). The total number of individuals planted in Chile was 870 and in Spain was 1740. The non-targeted surface vegetation was continuously removed over the experimental period by hand to ensure plants in both common gardens experienced similar levels of competition. No additional treatment, such as fertilisation, occurred in any of the common gardens.

Functional traits

The experiment lasted for 180 and 250 days at the Chilean and Spanish common gardens, respectively. At each common garden, weekly values of precipitation and daily values of mean temperature were obtained from the meteorological stations that were located closest to the experiments (i.e. Cauquenes INIA meteorological station: 35°57’S, 72°17’W; 164 m a.s.l. in Chile and Madrid Ciudad Universitaria meteorological station: 40°27’N, 3°43’W; 640 m a.s.l. in Spain; see graphs in Fig. 1 and the Suppl. material 3: Figure S3 for detailed meteorological data).

Plant survival and phenology were recorded three times a week from sowing to flowering and every two days from flowering to plant fructification. Plants that died prior to accomplishing fruit maturity were no longer employed for assessing performance traits, while plants that accomplished maturity were considered dead after reaching 75% senescence. Phenological observation included the date when each individual got the first floral bud and was used to calculate the days from planting to flowering.

The number of flower heads per plant was counted for every individual. Flower heads were collected after they had produced fruits but before the infructescence opened, to ensure we captured all seeds and avoided propagules spreading. The average number of fruits per flower head was calculated for each individual by averaging the number of
fruits counted over five flower heads that were collected from each plant when it reached around 50% senescence. The total seed output per plant was estimated by multiplying the average number of fruits per flower head by the number of flower heads per plant.

Once each individual had reached around 75% senescence, plants were harvested. Flower heads were removed and then the vegetative part was oven-dried at 60 °C for 72 hours. Afterwards, aboveground dry biomass (hereafter biomass) was weighed.

Due to the high mortality rate of *H. glabra* and *T. glomeratum* in the Spanish trial, further comparisons of phenology and performance traits between ranges (common gardens) were only possible to assess for *L. saxatilis*.

**Data analyses**

All analyses were performed in R v 3.2.3 (R Core Team 2015). To check differences in survival rates associated with climatic conditions of both common gardens, the cumulative survivals of the three species, expressed by their Kaplan-Meier curves, were plotted taking into consideration the environmental conditions of each common garden. For each species, comparisons for populations of both countries of origins (Spanish vs. Chilean) between Kaplan-Meier curves from the time seedlings were sown were performed with the R package survival (Therneau 2015).

We used mixed effects models using the base stats package plus lme4 (Bates et al. 2014) to explore differences in phenological and performance traits of *L. saxatilis*, *H. glabra* and *T. glomeratum* in the trials at both the native and the introduced ranges.
T. glomeratum associated with the country of origin of the populations. We considered the plant individual as the unit of analysis (L. saxatilis: n = 340; H. glabra: n = 186; T. glomeratum: n = 268). Models were fitted taking into account phenology (i.e. days to flowering), plant growth (i.e. dry aboveground biomass) and estimated seed output per plant as response variables. We used mixed effects models with a Gaussian error distribution for the three response variables. Fixed effects included the country of origin (Spain and Chile) and the precipitation on the population (as populations were selected along a rainfall gradient) for H. glabra and T. glomeratum. In the case of L. saxatilis, we also explored whether phenology and performance traits of this species varied between common gardens located in the native and in the introduced ranges; thus not only the previous fixed factors but also the range where the common garden was emplaced were included. The subplot where populations were planted in the common garden was included as the random effect nested within population. All the possible models, including origin and precipitation (and range in the case of L. saxatilis) as predictors (as well as their interactions), were computed.

We compared the possible models differing in the structure of fixed effects fitted by maximum likelihood. We calculated the Akaike Information Criterion corrected for small sample size (AICc). We selected the best-fit models (lowest AICc presenting differences in their AICc lower than 2; Burnham and Anderson 2002) employing the AICcmodavg package (Mazerolle 2015). The parsimony principle was applied on the subset of best models based on AICc and the model with the lowest number of parameters was chosen for subsequent analyses (Cox et al. 2006). Selected models were fitted by Restricted Maximum Likelihood and significant values for fixed effects were calculated with a type-III ANOVA analysis with the lmerTest package (Kuznetsova 2017). Model validation of the best-fit model was based on visually assessing the normality of residuals. To test over-dispersion, we checked that the residual deviance was lower than the residual degrees of freedom (Zuur et al. 2009).

In order to evaluate whether a delay in phenological development could entail an increase in plant performance, we also performed mixed-effects models for performance traits (biomass and seed output) in which we entered days to flowering as predictor, precipitation as co-variable and subplot where populations were planted in the common garden nested within the population as random effects. These models were performed by splitting the plant individuals by origin (i.e. Spanish and Chilean). Marginal r coefficients of these relationships as well as of the relationships between precipitation and phenology and performance traits were obtained per country of origin employing the R package MuMIn (Barton 2018). Finally, outliers that exceeded three times the inter-quartile range were removed prior to analyses, which only occurred for 1.5% of cases.

Results
There were differences between the climatic conditions of both Mediterranean regions; rainfall gradient was broader and number of months with water deficit longer in Chile than in Spain (300–1200 mm vs. 450–950 mm and 4–8 months vs. 3–4 months, respectively; Table 1).
The cumulative survivals of the three species, expressed by their Kaplan-Meier curves, were clearly different at both ranges, being significantly lower in the native range (Spanish trial) than in the introduced range (Chilean trial) (Fig. 1a–c, Suppl. material 4: Figure S4). In the Spanish trial, *H. glabra* and *T. glomeratum* – and, to a lesser extent also *L. saxatilis* – showed an abrupt mortality after 100 days from planting, whereas in the Chilean trial, the cumulative survival remained high (around 90%) until the end of the experiment. The high mortality in the native range could be related to the scarce precipitation during late autumn and winter (from 1 December to 22 March; see Fig. 1d–e and Suppl. material 3: Figure S3). Due to the high mortality of *H. glabra* (84%) and *T. glomeratum* (94%) in the Spanish common garden, the comparison of phenology and performance traits between ranges (common gardens) was only possible for *L. saxatilis*.

According to the generalised linear mixed-effects models, the factors that explained most of the variation of phenology and performance traits for different populations varied amongst species (Table 2). For *T. glomeratum*, both origin of and precipitation on the population had a significant effect on days to flowering, that being the phenological development was significantly longer for Spanish populations than for Chilean ones (days to flowering for Spanish populations: 142 ± 3; days to flowering for Chilean populations: 131 ± 3). The relationship between days to flowering and the precipitation on the population were significant, regardless of the country of ori-

<table>
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<tr>
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<th>T. glomeratum</th>
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gin (Spanish populations: $r = 0.53; P < 0.05$; Chilean populations: $r = 0.28; P < 0.05$; Fig. 2) In the case of *H. glabra*, the country of origin and its interaction with precipitation had an effect on the days to flowering; there was a close and positive relationship between days to flowering and the precipitation on the population for the Spanish populations ($r = 0.69; P < 0.05$) but not for the Chilean ones (Table 2; Fig. 2). For *T. glomeratum* and *H. glabra*, differences in days to flowering between the most precocious populations (from the driest provenances) and the latest flowering ones (from the wetter provenances) were up to 27 days. For *L. saxatilis*, the interaction between range and origin (model coefficient for the interaction origin(Spanish)*range(native): $t = -6.59; p < 0.001$) had a significant influence on phenology (i.e. days to flowering) in the native range, while the effect of precipitation on the population origin was only significant for Spanish populations grown in the native range (model coefficient for the interaction origin(Spanish)*precipitation*range(native): $t = 5.14; p < 0.001$; Table 2; Fig. 2). Contrary to what was expected, no significant relationships were found between days to flowering and both biomass and seed output ($P > 0.05$) for any species considered, indicating that a longer time for development does not involve greater reproductive effort.

For *T. glomeratum* and *H. glabra*, the biomass was only determined by the country of origin, with significantly larger plants coming from native populations (*T. glomeratum:*
Spanish populations: 11.8 g ± 0.8 g; Chilean populations: 8.7 g ± 0.5 g; *H. glabra*: Spanish populations: 33.3 g ± 4.2 g; Chilean populations: 19.5 g ± 1.8 g; Table 2; Fig. 2). However, for *L. saxatilis*, there was a negative relationship between biomass and precipitation regardless of the origin of the populations considered (Fig. 2).

Seed output displayed by *T. glomeratum* and *H. glabra* was only determined by the country of origin, with native populations displaying greater number of seeds (*T. glomeratum*: Spanish populations: 8978 ± 1106; Chilean populations: 5525 ± 320; *H. glabra*: Seed output: Spanish populations: 14686 ± 2142; Chilean populations: 7500 ± 1545; Table 2; Fig. 2). For *L. saxatilis*, there was a negative relationship between seed output of native populations and precipitation on the population (Fig. 2).

Common garden comparisons showed that all the studied parameters were mainly influenced by range. Hence, phenology was significantly shorter in the introduced range than in the native one; while biomass and seed output were significantly greater in the introduced range than in the native one (Fig. 3).

**Discussion**

The need to carry out comparative studies of native versus introduced populations in order to detect key aspects to explain the invasion success as those related with functional traits of invaders has been highlighted in the scientific literature (Bossdorf et al. 2005, Molina-Montenegro et al. 2010, 2011, Lemoine et al. 2016). In this sense, our study highlights the differences existing in performance traits and especially in plant phenology associated with the invasion process of three herbaceous plants native to Spain but invasive to Chile.

However, comparison between native and introduced ranges was only possible for *L. saxatilis* due to the high mortality of *H. glabra* and *T. glomeratum* in the Spanish common garden. The three species presented a similar survival curve in the introduced range, where the weather conditions during the common garden experiment were milder and more benign. In this sense, the high survival rate showed by *L. saxatilis* in the native range, regardless of the extreme weather conditions during the Spanish common garden experiment and its resilience after a major drought event (see Fig. 1) could itself constitute an indicator of the plasticity of this species. However, please note that our results regarding phenotypic plasticity should be carefully interpreted as we could not account for genetic distances between mother sources and inter-population gene flow.

The phenology of *L. saxatilis* was mainly influenced by range instead of by country of origin of the populations; thus days to flowering showed different responses for the same population (either native or exotic ones) under different environmental conditions (native vs. introduced range). The variation in *L. saxatilis* phenology between ranges reflects its great capacity to acclimatise to changing environmental conditions (Geng et al. 2007, Gratani 2014). The delay in time to flowering of both Chilean and Spanish populations in the native range (Spain) can be attributed to the lower temper-
atures in autumn and spring and lower precipitation compared to the introduced range (Chile) (Fig. 1 and Suppl. material 3: Figure S3). It is known that higher temperatures (Bradley et al. 1999) and longer day length accelerates plant development in temperate species (Molina-Montenegro and Naya 2012) and differences in day length and night length become more extreme at higher latitude (Bradshaw and Holzapfel 2008). Thus, in Madrid (latitude 40°26’N), the day length or photoperiod is shorter in autumn and winter, but longer in spring and summer than in Cauquenes (latitude 35°58’S). These patterns in temperature and photoperiod gave rise to large differences in the length of the growing season observed between the native and the introduced range.

Changes in flowering phenology amongst different populations constitute an indicator of ecotypic differentiation to the environmental conditions of the provenances where populations originated. According to our findings, populations of *T. glomeratum* and *H. glabra* have mainly undergone variation in their phenology to acclimatise to the new environmental conditions. These species exhibited clear differences in their phenology associated with the country of origin of the populations. In the case of *T. glomeratum*, phenological development was shorter for populations (both native and exotic ones) originating in drier provenances and phenology became lengthened for populations originating in more humid provenances (Table 2; Fig. 3). In fact, similar results were reported by del Pozo et al. (2000, 2002a, 2002b) in another Fabaceae, *Medicago polymorpha*. Regarding *H. glabra*, a similar delay was observed in phenological development for Spanish populations originating in more humid provenances, but this trend was not observed for Chilean populations. The fact that exotic populations of *T. glomeratum* originating in more humid provenances showed a phenological delay in relation to those originating in drier provenances (although this was not shown for exotic populations of the two daisies) could be related with the time since introduction of the three invasive species in central Chile. The leguminous *M. polymorpha* and *T. glomeratum* were first recorded before 1799 and in 1897, respectively (Castro et al. 2005), so they had been naturalised in the introduced range for several decades, possibly with enough time to undergo acclimatisation. Following the same criteria, *H. glabra*, a species that was first recorded in Chile in 1905 (Fuentes et al. 2013), showed differences between native and exotic populations; nevertheless, Chilean populations did not show ecotypic differences amongst them. In contrast, *L. saxatilis* was first recorded in Chile in 1963, therefore it has had only a short time to undergo rapid evolution (Buswell et al. 2011) or to develop further strategies, relying mainly in plastic responses in its phenology and performance traits. However, the significant interactions between range, country of origin and precipitation found in *L. saxatilis* point to ecotypic differences for Spanish populations grown in the native range, where populations from drier provenances have shortened their period of phenological development.

In any case, contrary to what might be expected, the delay in phenology associated with the precipitation on the population showed by *T. glomeratum* and by the native populations of *H. glabra* was not adaptive *sensu stricto* as it did not increase the performance traits of these species. Therefore, this mechanism could allow populations to acclimatise to a wider environmental range (i.e. enhance their invasiveness via increas-
Figure 3. Comparisons between trials at the native and the introduced ranges for native and exotic populations of *Leontodon saxatilis*. Graphs show mean values and standard errors of days to flowering (a), biomass per plant (b) and seed output per plant (c) grouped by origin of the population. Percentages of variation between the native trial and the invasive one are also shown. The arrow indicates the direction of the colonisation process, from the source to the recipient region.
ing range expansion) but it did not increase plant growth (i.e. biomass) nor propagule pressure (i.e. seed output did not result in enhanced days to flowering). Similarly, the delay in *L. saxatilis* phenological development at the native range was not invested in producing more biomass or displaying more seed output, probably due to the lower precipitation at the trial located in the native range compared to the trial at the introduced range. In the case of this species, no consistent patterns were found associated with the country of origin of the populations.

Regarding performance traits, *T. glomeratum* exhibited clear differences in their biomass and seed output displayed associated with the country of origin of the populations. Contrary to what we expected, exotic populations have not apparently undergone selection for traits that allowed them to outperform native populations of the same species; in fact, native populations displayed greater seed output when cultivated under common garden conditions in the introduced range (Table 2). The very same trend was observed for *H. glabra* and *L. saxatilis*. This can be related to the fact that native populations of the three species showed a much lower survival rate than exotic ones under the novel environmental conditions of the introduced range (see Suppl. material 4: Figure S4). Exotic populations of these species might have overcome greater hydric stress typical from the Mediterranean-type region of central Chile by evolving resistance mechanisms, (presumably costly) which in turn trade off against biomass and seed output. Performance traits for *L. saxatilis* were mainly influenced by range (see Martín-Forés et al. 2017c for further discussion); in this sense, the increase in biomass and seed output displayed by *L. saxatilis* in the introduced range compared to the native one, especially highlighted for native populations, reflects the invasive ability of this species, which shows an enhanced propagule pressure in the introduced range and the capacity to spread there. Our findings support the invasion patterns of *L. saxatilis* in central Chile, as it is the most frequent exotic species in this region (Martín-Forés et al. 2012) and it is widely distributed due to its invasiveness (Martín-Forés et al. 2015, 2017c). It is also an invader in other Mediterranean regions such as California and southern Australia (Groves et al. 2003, DiTomaso et al. 2007); thus, such a great plastic response might raise the potential of this species to spread in a global changing scenario (Guerin et al. 2014).

Their particular dispersal pathways could also influence these differences identified amongst species. For instance, *Trifolium glomeratum* has animal-dispersed fruits with low spreading capacity, probably needs to rely more on acclimatising to local conditions and adjusting its phenological development in relation to the precipitation on the origin of the population. On the contrary, both *H. glabra* and *L. saxatilis* have fruit dimorphism (i.e. heterocarpy; Baker and O’Dowd 1982, Brändel 2007); they are not only animal-dispersed but also undergo long distance dispersal events by wind (Martín-Forés et al. 2017c, 2018); in this case, a plastic response in survival, phenology and performance traits could be the most successful mechanism in the novel environment. However, further detailed research would be necessary to elucidate whether different mechanisms operate in the acclimatisation process to a new environment depending of the dispersal pathway of the species.
Conclusion

Overall, the studied invasive species have evolved in their native range for millennia, while in their introduced range, they have only been present for few decades or over the last few centuries. Once they arrived to Chile, they spread and adapted to the whole Chilean climatic gradient. *Trifolium glomeratum* and *H. glabra* mainly relied on ecotypic differentiation for plant phenology associated with the population origin while *L. saxatilis* mainly showed plasticity when growing in different ranges. However, changes in phenology were not reflected in greater biomass or seed output display but might rather be related to range expansion processes. Despite relying on different strategies, all these species have resulted as successful invaders in the Mediterranean Biome. All this highlights that, not only performance traits, but also phenology and plant survival are key traits that need to be targeted to account for species invasiveness and therefore to predict future invasions and control for existing ones.

Acknowledgements

We thank the Spanish Ministry of Science and Innovation for the financial support received to carry out this study (CGL2009-08718) and the grants REMEDINAL (S2013/MAE-2719 REMEDINAL3-Comunidad de Madrid) and SPONFOREST (BiodiVerSa3-2015-58, PCIN-2016-055) and the Spanish Ministry of Education, Culture and Sport, because of the pre-doctoral FPU scholarship of the main author (AP2009-0518). We thank the State Meteorological Agency for providing meteorological data (AEMET, http://www.aemet.es/es/portada). We are especially grateful for the advice and suggestions provided by Greg Guerin. We would like to acknowledge Teresa Aravena, María Elena Díaz, Teresa Moreno Vicente, Marta Avilés, Devayana Valero and Ricardo Prentice for their support in phenological observations and Laura Sánchez-Jardón and Carlos Ovalle for field support. Likewise, we would like to acknowledge the whole INIA-Cauquenes Institution, in central Chile and the team from the Faculty of Agronomy of the Polytechnic University of Madrid, especially Daniel de la Torre Llorente.

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Supplementary material 1

Figure S1
Authors: Irene Martín-Forés, Miguel A. Casado, Isabel Castro, Alejandro del Pozo, Marco A. Molina-Montenegro, José M. de Miguel, Belén Acosta-Gallo
Data type: occurrence
Explanation note: Distribution of *Leontodon saxatilis*, *Hypochaeris glabra* and *Trifolium glomeratum* in both the native (Spain) and the introduced (Chile) ranges.
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Link: https://doi.org/10.3897/neobiota.41.29965.suppl1

Supplementary material 2

Figure S2
Authors: Irene Martín-Forés, Miguel A. Casado, Isabel Castro, Alejandro del Pozo, Marco A. Molina-Montenegro, José M. de Miguel, Belén Acosta-Gallo
Data type: occurrence
Explanation note: Map of the studied areas of Mediterranean grasslands in Spain and Chile, including populations sampled following a rainfall gradient (see Table 1). The location of the common gardens is shown (x). This figure has been adapted from Martín-Forés et al. (2015, 2018).
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Link: https://doi.org/10.3897/neobiota.41.29965.suppl2
Supplementary material 3

Figure S3
Authors: Irene Martín-Forés, Miguel A. Casado, Isabel Castro, Alejandro del Pozo, Marco A. Molina-Montenegro, José M. de Miguel, Belén Acosta-Gallo
Data type: species data
Explanation note: Daily maximum and minimum temperatures (A and B) and precipitation (C, D) at Cauquenes, Chile (A, C) and Madrid, Spain (B, D). Data are from 1 January – 31 December 2011 in Chile and 1 July 2011 – 30 June 2012 in Spain. The arrows indicate transplanting dates.
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Link: https://doi.org/10.3897/neobiota.41.29965.suppl3

Supplementary material 4

Figure S4
Authors: Irene Martín-Forés, Miguel A. Casado, Isabel Castro, Alejandro del Pozo, Marco A. Molina-Montenegro, José M. de Miguel, Belén Acosta-Gallo
Data type: statistical data
Explanation note: Tree diagrams for *Leontodon saxatilis*, *Hypochaeris glabra* and *Trifolium glomeratum* showing significant differences in survival curves. Each diagram represents the comparison of Kaplan-Meyer curves considering common garden range (first level: introduced vs native), country of origin (second level: Chile vs Spain), and populations (third level: nomenclature as in Table 1). For each population the percentage of survival is shown and the lowercase letters indicate similar groups amongst populations.
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Link: https://doi.org/10.3897/neobiota.41.29965.suppl4