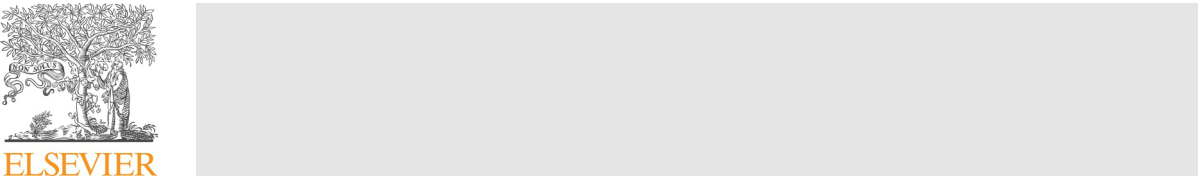
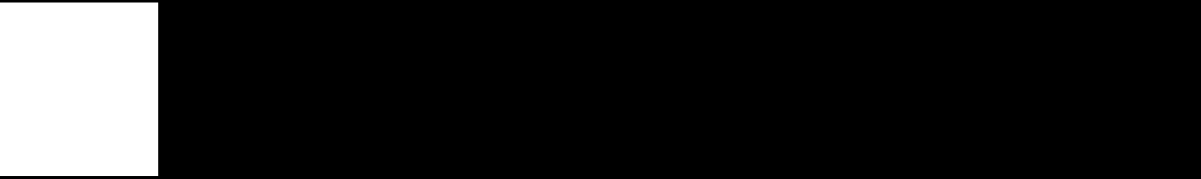
[Environmental and Experimental Botany 176 (2020) 104103](https://doi.org/10.1016/j.envexpbot.2020.104103)



Contents lists available at [ScienceDirect](http://www.sciencedirect.com/science/journal/00988472)



Environmental and Experimental Botany

journal homepage: [www.elsevier.com/locate/envexpbot](https://www.elsevier.com/locate/envexpbot)

Two sides of the same coin: Does alien *Impatiens balfourii* fall into an ecological trap after releasing from enemies?

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ARTICLE INFO

*Keywords:*

Enemy release

Altitude

Ornamental plants

Resources

Re-Allocation

Replacement species

Self-compatibility

ABSTRACT

The evolution of increased competitive ability (EICA) hypothesis assumes that recently introduced populations of alien species are under low pressure from enemies, which allows them to reduce investment into defence and re-allocate the saved resources into the improvement of characteristics related to competitive abilities. We performed EICA tests to check if the low dispersal potential of *Impatiens balfourii* in Europe results from a limited evolution of its capability to release from enemies or from the low phenotypic plasticity of this trait.

We tested habitat preferences of the species and compared the levels of enemy pressure in different habitats. This allowed us to determine if the tendency to occur along roadsides is manifested at the European scale in this species and if this preference is determined by a lower pressure of enemies in that habitat. It cannot be ruled out that occurrence along roads can be maladaptive for the species, and this habitat can potentially constitute an ecological trap due to frequent mowing.

The study was carried out in six European populations differing in age and in five types of habitat. Enemy pressure was estimated by assessing leaf damage in adult plants and fungal pathogen load at the seed stage. We tested if the saved reserves are invested in the size and/or fertility of adult plants or in the seed quality. Activity of pollinators in different populations and habitats was also assessed.

The majority of the results for adult plants indicate that the younger populations of *I. balfourii* release from enemy pressure better than the older ones. However, we found only weak support that the reserves previously spent on defence against natural enemies could be re-allocated into larger size or increased fertility. Only in the youngest population from Andorra was it largely confirmed. However, at seed stage, we found no support for the EICA assumptions in any of the populations. Thus, in general, the results of our study provide a fairly weak support for the EICA hypothesis.

In comparisons between the habitats, we found that in five of the six populations, the species preferred roadsides, which was probably determined by the lower enemy pressure there. However, it cannot be excluded that this preference can be maladaptive and that this habitat can potentially constitute an ecological trap due to frequent mowing. Notably, the species was also abundant along streams, where the plants are less disturbed by human activities. This finding was particularly pronounced at the invasion forefront in Andorra, where the species was completely absent from roadsides. Therefore, we assume that unless control measures are under-taken, streamsides may be more important for the future invasion of *I. balfourii* than roadsides in that area.

The ornamental value of *I. balfourii* makes it an obvious candidate to replace its banned invasive counterpart *I.* *glandulifera* in trade. The increase in popularity in trade and cultivation would inevitably lead to an increase inpropagule pressure, which could be crucial for increasing the rate of invasion of *I. balfourii* on a large scale in Europe.

**1. Introduction**

The assumption positing that invasive alien species are successful because they leave behind their natural enemies (e.g., herbivores,



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predators, parasites or pathogens) in the native range was formulated as the enemy release hypothesis (ERH) (Elton, 1958; Keane and Crawley, [2002](#page13); Maron and Vilà, 2001). Numerous studies have positively ver-ified this hypothesis (Adams et al., 2020; Cincotta et al., 2009; [Engelkes](#page12)

<https://doi.org/10.1016/j.envexpbot.2020.104103>

Received 2 March 2020; Received in revised form 29 April 2020; Accepted 30 April 2020

Available online 13 May 2020

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et al., 2008; Mitchell and Power, 2003; Vilà et al., 2005), although there are also studies whose results indicate the opposite, showing that some alien species are not able to release from enemy attacks after their introduction into new areas (Agrawal and Kotanen, 2003; Gross et al., [2001](#page12); Parker and Hay, 2005; Schultheis et al., 2015; van Kleunen and Fischer, 2009). However, changes in enemy release abilities with time after introduction, as a result of the evolutionary processes or pheno-typic plasticity, have not been thoroughly studied.

Today, it seems obvious that evolution of different life history traits facilitates expansion of an alien species into regions that were pre-viously inaccessible and establishment in regions that used to be too hostile for its survival and/or reproduction (Gruntman et al., 2017; Niklas, 2016; Walther et al., 2009). The evolution of increased com-petitive ability (EICA) hypothesis (Blossey and Notzold, 1995) takes an evolutionary perspective on the enemy release capability. This hy-pothesis assumes that as the enemy pressure on newly introduced po-pulations of alien species is low, individuals may reduce their invest-ment into defence against enemies and re-allocate the saved resources into the improvement of competitive abilities. This may result in the superiority of the introduced (young) populations of alien species over the co-occurring native species in terms of acquiring, retaining and exploiting resources. Consequently, this leads to an increase in the abundance of alien species, their spread and, finally, their impact in the initial phase of the invasion. However, subsequently, in the older po-pulations of alien species, the enemy release potential decreases over time, which results in decreased allocation to competitive abilities. Therefore, the older populations are expected to be less competitive than the younger ones (Gioria and Osborne, 2014; Gruntman et al., [2017](#page12); [Siemann et al., 2006](#page13)).

The EICA hypothesis was tested on a number of alien plant species (Siemann et al., 2016; Siemann and Rogers, 2003; Uesugi and Kessler, [2013](#page13)), including *I. glandulifera* (Gruntman et al., 2017) in which the level of leaf damage in younger populations was significantly lower than that in older ones. However, the concentration of allelopathic compounds, which may increase enemy resistance (Gruntman et al., [2017](#page12); Mitchell et al., 2007), did not differ between those populations. Thus, it was not confirmed that enemy release abilities and allelopathic potential changed over time in parallel. Unfortunately, those studies did not focus on the performance of the plants; therefore, it is not known if better release from the enemies in the younger populations is translated into better conditions of the surveyed plants.

It is also known that organisms may alter their phenotype in re-sponse to the environmental conditions in which they develop, known as phenotypic plasticity (Agrawal, 2001). The high phenotypic plasti-city in traits of the successful invaders may allow them to maintain high fitness even in unfavorable environments (Davidson et al., 2011; Richards et al., 2006). This advantage is also translated into a higher tolerance of such species against biotic and abiotic conditions in newly established environments.

In the present study, enemy release tests were carried out on *I.* *balfourii*. This species is in a close taxonomic relationship with *I. glan-dulifera* (Janssens et al., 2009); however, the two species significantlydiffer in their level of invasiveness. *I. balfourii* is invasive only locally in France, Italy, and Croatia (Najberek et al., 2018), while its relative is one of the most prominent invasive alien species throughout Europe (Drake, 2009). Interestingly, both species are characterized by high attractiveness for pollinators, self-compatibility, no effect of inbreeding depression and high reproductive capacity (Jacquemart et al., 2015; Ugoletti et al., 2011). Both also show high allelopathic potential (Vrchotová et al., 2011) and have antimicrobial and antioxidant properties (Szewczyk et al., 2016). Therefore, determining the factors suppressing *I. balfourii* invasion may yield insights into the mechanisms leading to high invasiveness, which is important to theoretical aspects of biological invasions as well as to the development of practical measures to mitigate their impacts (Gioria and Osborne, 2014; [Ugoletti](#page13) et al., 2011; van Kleunen et al., 2010).

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One of the factors indicated to limit the invasive potential of *I.* *balfourii* in Europe is its low tolerance to spring frosts (Tabak and vonWettberg, 2008). However, because young populations of *I. balfourii* are well-established in montane areas, e.g., in Andorra, we assumed that its tolerance to spring frost is sufficient enough to consider this factor as increasingly less limiting. Moreover, Perrins et al. (1993) found that seedlings of *I. glandulifera* and *I. balfourii* were more resistant to a heavy frost (−5 °C) than seedlings of *I. parviflora* – another invasive alien representative of this genus in Europe (Roy et al., 2019). Another possible reason for the low invasiveness of *I. balfourii* is its low popu-larity as an ornamental, which limits its propagule pressure (Adamowski, 2009). It cannot be excluded, however, that due to its ornamental potential, the trade of this species may increase in the fu-ture, particularly taking into account that in 2017, the trade in the si-milar looking invasive *I. glandulifera* was stopped by EU legislation (European Commission, 2017), thus making *I. balfourii* an obvious candidate to replace it.

We concentrated on disentangling the role of the third possible limiting factor that we identified in our earlier studies (Najberek et al., [2017](#page13)), namely, maladaptive habitat preferences. Having studied two populations from Ticino (Switzerland) and Lombardy (Italy), we con-cluded that the preference of *I. balfourii* for roadsides may be associated with a lower pressure of enemies there. At the same time, however, roadsides constitute a non-optimal habitat due to intensive mowing that prevents the species from spreading; this process may act as an ecolo-gical trap for the species. As those studies included only two popula-tions, the results may not be representative of the whole of Europe. Moreover, the temporal aspect was not considered earlier, as the stu-died populations were of similar age.

The post-introduction changes of enemy release capability that *I.* *balfourii* has been undergoing after its introduction to Europe may befar from parallel to those of *I. glandulifera*. Therefore, the aim of the present study was to test the temporal aspect of enemy release ability of *I. balfourii* as a result of evolutionary and/or phenotypic plasticity. Thetests were conducted including adult plants as well as their seeds. Because of the low invasiveness, we expected the younger populations of the species to be under the same enemy pressure as the older ones, which, according to EICA assumptions, would not allow them to save resources on defence and reallocate them into larger size and fertility at the early stages of invasion. Enemy pressure would therefore be a limiting factor that may explain the low invasive potential of *I. balfourii* in Europe.

In addition, in tests, the habitat preferences of the species in po-pulations differing in age were checked, and the levels of enemy pres-sure in those habitats were compared. This allowed us to determine (1) if the species’ tendency to occur along roadsides is manifested on a European scale, and (2) if such preferences are determined by a lower pressure of enemies in that habitat. The importance of such maladap-tive habitat preferences may depend on differences in road main-tenance regimes in particular countries and is most pronounced in the case of annual plants that disperse only with seeds – such as the *Impatiens* species. Ecological trap can therefore be a factor significantlyweakening the spread of *I. balfourii* in Europe.

Moreover, we examined the activity of pollinators visiting the sur-veyed plants. We expected that enemies and pollinators have a com-bined effect on the plant performance. For instance, the enemies may decrease expenditure of resources into flower development, which should result in lower pollinator activity, or else, the attacked plants may be stimulated to produce more attractive flowers to survive, which in turn would increase pollinator activity.

**2. Methods**

*2.1. Species selected for study*

*Impatiens balfourii* is an annual plant species introduced into Europe

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**Table 1**

The surveyed localities of *Impatiens balfourii* with habitat category (R – roadside, Ra – ruderal area, S – streamside, Ua – urban area, Uga – urban green area), elevation, geographical coordinates in decimal degrees format (X – longitude, Y – latitude), and date of testing of the increased competitive ability (EICA) hypothesis.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Population | Habitat description | Habitat type category | Elevation | Coordinates |  |  | Date of EICA tests |  |
|  |  |  |  |  |  |  |  |  |
|  |  |  |  | X | Y | |  |  |
|  |  |  |  |  |  | |  |  |
| Le Rozier | Bank of La Jonte river (∼10 m broad) in Le Rozier | S | 381 | 3.20867 | 44.19022 | | 2018.10.9−10 |  |
|  | Bank of Tarn river (∼50 m broad) in Mostuéjouls | S | 380 | 3.17116 | 44.20149 | |  |  |
|  | Roadside in Boyne | R | 382 | 3.159973 | 44.2011 |  |  |  |
| Torino | Roadside in Mostuéjouls | R | 481 | 3.185119 | 44.20326 | | 2018.09.20−22 |  |
| Between paths in Park area in Venaria Reale (near Parcheggio Castellamonte) | UGA | 246 | 7.627409 | 45.13757 | |  |
|  | Along the Ceronda river in Venaria Reale | S | 244 | 7.629059 | 45.1367 |  |  |  |
|  | Along city channel in Venaria Reale (near Viale Giuseppe Mazzini street) | S | 245 | 7.629387 | 45.13538 | |  |  |
| Insubria | Roadside in Superga | R | 658 | 7.770613 | 45.07864 | | 2018.09.14−17 |  |
| Bank of Giona river in Maccagno | S | 218 | 8.744315 | 46.04364 | |  |
|  | Bank of Giona river in Maccagno | S | 247 | 8.753056 | 46.04419 | |  |  |
|  | Roadside along Corso Europa street in Maccagno | R | 249 | 8.739763 | 46.10324 | |  |  |
|  | Roadside in Luino | R | 324 | 8.76859 | 45.99976 | |  |  |
| Istria | Roadside in Germignaga | R | 286 | 8.716957 | 45.9866 |  | 2018.09.9−10 |  |
| Ruderal area near buildings in Lanišće | RA | 543 | 14.11604 | 45.4071 |  |  |
|  | Ruderal area near buildings in Vodice | RA | 666 | 14.0536 | 45.4842 |  |  |  |
|  | Roadside in Vodice | R | 663 | 14.05327 | 45.48417 | |  |  |
| Zagreb | Roadside in Vele Mune | R | 643 | 14.15805 | 45.45773 | | 2018.09.5−7 |  |
| Bank of montane stream (∼2 m broad) in Medvednica Nature Park | S | 363 | 15.9482 | 45.86551 | |  |
|  | Bank of montane stream (∼1 m broad) in Medvednica Nature Park | S | 312 | 15.9432 | 45.85674 | |  |  |
|  | Along dried ditch in Park Maksimir | UGA | 131 | 16.01495 | 45.82215 | |  |  |
|  | Between paths in Park Maksimir | UGA | 164 | 16.01973 | 45.83878 | |  |  |
|  | Roadside near Park Maksimir | R | 162 | 16.02136 | 45.83773 | |  |  |
| Andorra | Roadside near Park Maksimir | R | 158 | 16.02132 | 45.83794 | | 2018.10.4−6 |  |
| Near buildings and parking space in Les Escaldes | UA | 1067 | 1.537886 | 42.51338 | |  |
|  | Near buildings and parking space in Les Escaldes | UA | 1070 | 1.541513 | 42.51118 | |  |  |
|  | Near buildings and parking space in Sant Julià de Lòria | UA | 940 | 1.49406 | 42.47045 | |  |  |
|  | Bank of montane stream (∼5 m broad) in Les Escaldes | S | 1092 | 1.546181 | 42.51112 | |  |  |
|  | Bank of montane stream Riu d'Os (∼2 m broad) in Sant Julià de Lòria | S | 916 | 1.489015 | 42.47589 | |  |  |
|  | Bank of montane stream El Runer (∼2 m broad) near Gatera Oficial D'Andorra | S | 856 | 1.474662 | 42.43525 | |  |  |
|  |  |  |  |  |  |  |  |  |

in 1901 from the western Himalayas (Fournier, 1952; Nasir, 1980). In Europe, the species occurs in areas altered by humans (e.g., roadsides, touristic trails, urban green areas, ruderal areas) and in seminatural or natural sites, such as wet meadows, woodland margins, riversides, scrub and forest edges (Jacquemart et al., 2015; Najberek et al., 2018). The species is very attractive for pollinators, and its reproductive po-tential is high. It has pink and white flowers and disperses its seeds ballistically (Jacquemart et al., 2015). In Europe, flowering occurs from June to October (Jacquemart et al., 2015), and in most regions, the seed set starts in late August (Najberek et al., 2020). In Europe, *I. balfourii* is usually recorded at elevations below 1000 m a.s.l., whereas in its native Himalayas, it grows at 1500–2500 m a.s.l. (Adamowski, 2009; [Nasir,](#page13) [1980](#page13)).

*2.2. Surveyed populations*

The present study is based on data collected in 2018 in six European populations of *I. balfourii*: Zagreb, Istria, Insubria, Torino, Andorra and Le Rozier (Table 1, Fig. 1). The approximate times of the first local introduction were estimated for five of the six surveyed populations (Fig. 1), and only the Le Rozier population remains ambiguous in this respect, despite exploring several references (INPN, 2019; Si Flore, [2019](#page13); Tela Botanica, 2019) and consulting with local experts (see Ac-knowledgements). The earliest information on the species in this area comes only from the 1970s (Jouret, 1977). However, as in the neigh-boring Montpellier area, the species had been reported in 1906, and we assumed that it was introduced into Le Rozier in the first half of the 20th century ([Fig. 1](#page4)).

Le Rozier, Torino and Insubria are referred to as ‘older populations’, while Istria, Zagreb and Andorra are referred to as ‘younger popula-tions’ throughout the text.

*2.3. Localities and data collection*

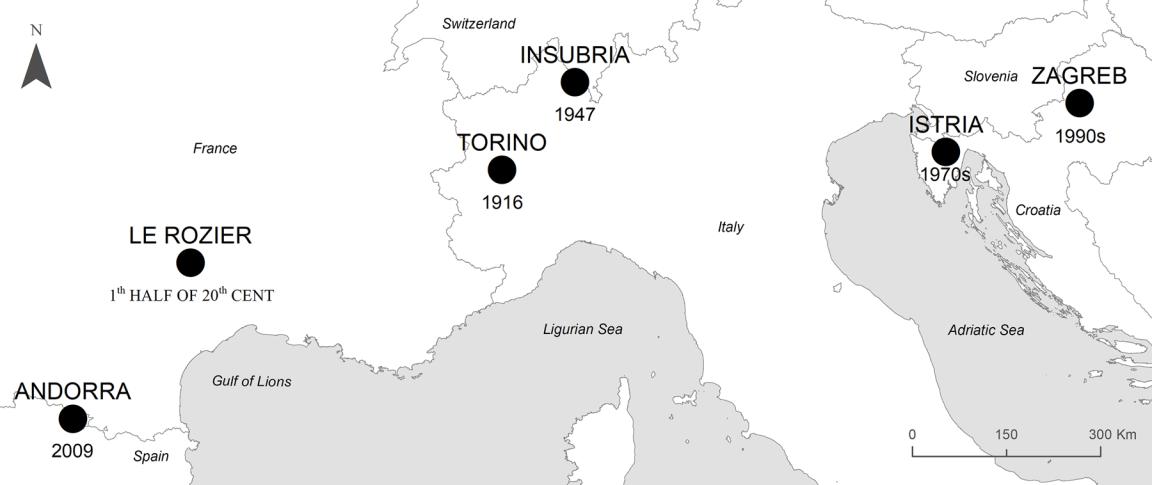
Surveys of each population were carried out during the flowering and seed set phase, when the species is easy to detect. The localities of the species were GPS-logged in each study region, and data on patch area and shading were also collected. A few of the detected localities (in each population), which were both representative for the species and located outside of private properties, were selected for further ex-amination. The selected localities and dates of EICA tests are included in Table 1. Moreover, the assumption of the study was to conduct the experiment in different habitats, with at least one repetition per habitat within each population. This assumption was not fulfilled only for urban green areas and roadsides from Torino (Table 1), as no more suitable localities were found. The habitats were classified according to our earlier studies ([Najberek et al., 2017](#page13)).

At each selected site, 10 individuals of *I. balfourii* were randomly selected from the pool of plants in the same development phase (flowering and seed set) and were individually marked for tracking in the further EICA examination. In total, 2900 individuals of the species were included in the tests. The examination was always carried out between 09:00 and 17:00 and by the same researcher. The survey days were warm, rainless and usually windless (maximal recorded wind speed = 2.7 m/s), with air temperature ranging from 20 °C to 31 °C. Wind speed and air temperature were measured twice during each survey (in the initial and final phases) with a hand-held environmental meter (Extech 45170CM). Sun radiation was also noted during each survey (classified as ‘sunny’, ‘sunny/cloudy’ or ‘cloudy’).

In each surveyed plant, stem height and diameter (near the ground) as well as the width and length of leaves were measured. In addition, 10 first leaves, counting top-down, were measured. To estimate fertility, the numbers of flowers (including buds), pods and mature seeds per plant were counted. In total, 20,551 flowers, 589 pods and 4612 seeds were counted. Moreover, sets of all mature seeds per plant were

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**Fig. 1.** Surveyed populations of*Impatiens balfourii*, with the date of the species introduction (Zagreb,Ilijanić et al., 1994; Istria, Slavko Brana, pers. comm. 2018;Insubria, Info Flora, 2019; Torino, Adamowski, 2009; Andorra, Atlas de la Flora de los Pirineos, 2019).

weighed (with 0.00001 g accuracy; scale model Radwag AS.62.R2). For each plant, the frequency of leaves showing damage symptoms

(including diseases; hereinafter ‘leaf damage’) was calculated, and the symptoms were categorized (e.g., as rusts and spots, deformations, mines or browsing; Appendix A). Moreover, collected mature seeds were stored in paper bags. They were later used in mycological analyses testing the pressure fungal pathogens exerted on *I. balfourii* seeds.

In addition, during the survey, pollinators visiting *I. balfourii* flowers were filmed (camcorder model: Sony HDR-CX240E). At each locality, the camera was installed near individuals selected for EICA tests and focused on flowers (their number was noted and included in the ana-lysis). The recordings were analyzed, and the recorded pollinators were identified to six groups of insects (*Apidae*, *Bombini*, *Diptera*, *Sphingidae*, *Syrphidae* and *Vespidae*). Only a few pollinators were identified at thespecies level. The length of each pollinator visit was also measured and included in the analyses as pollination time.

*2.4. Mycological analysis*

Laboratory analyses were carried out in the Department of Plant Protection, Wroclaw University of Environmental and Life Sciences. The methodology of mycological analyses followed the one that we used in our earlier studies (Najberek et al., 2018). Seeds collected from each individual plant were evenly divided into two groups. Seeds in the first one were left non-disinfected, while those in the second one were disinfected with 1% sodium hypochlorite solution for 5 s. The division allowed us to check if the cores of the seeds of the species were also affected.

Seeds from each individual plant, divided into non-disinfected and disinfected groups, were placed on petri dishes (one or two, depending on their number) filled with 2% solidified maltose. In total, 4612 seeds were used. If the number of seeds per plant exceeded 10, they were placed on an additional dish. The total number of dishes was 815.

Growing fungal colonies were identified at the species level based on morphological characteristics, including the type, colour and size of the mycelium. Microscope slides were prepared so that the spores could be measured, as their size is one of the most important characters for species identification (Pitt and Hocking, 2009; Watanabe, 2011). Co-lonies of fungal taxa in each petri dish were counted. Most frequently, either one colony (*N* = 548 cases) or none (*N* = 123 cases) grew on seeds; two or more colonies were recorded less frequently (*N* = 144 cases).

Because the potential negative impact of enemies on seeds was as-sessed, we classified the recorded fungal species as true pathogens or

secondary pathogens and used only the former group in enemy pressure tests. This group included obligatory pathogens with invariably nega-tive impacts on a plant and its seeds (de Wit, 2007; Najberek et al., [2018](#page13)). On the other hand, secondary pathogens are less harmful and, in some circumstances, their presence may even benefit a plant by limiting its infestation by true pathogens (Liggitt et al., 1997; Najberek et al., [2018](#page13)). The recorded bacterial colonies were also classified as true pa-thogens because they cause enzymatic decomposition of living and dead tissues, and the result of their occurrence on seeds is gangrene and death of the seedlings (Mansfield et al., 2012).

*2.5. Statistical analysis*

All statistical analyses employed SPSS version 26.0 (IBM Corp., [2016](#page12)). The data were analyzed with the use of generalized linear mixed models (GLMM). Linear models were used for interval target variables in almost all analyses. Only in one model – for the pollination time – used negative binomial regression for the numerical data. Pairwise contrasts were applied for comparisons between the studied popula-tions, habitats and groups of pollinators. Moreover, the arcsine trans-formations were used in linear models to normalize frequency data.

In the base model for leaf damage, the target variable was the arcsine transformed frequency of damaged leaves (calculation formula: ‘Leaf damage’ = ASIN (SQRT (number of damaged leaves/total number of leaves of the surveyed plant)). Covariates were the surveyed popu-lation (‘Population’), habitat type (‘Habitat’) and elevation (‘Elevation’). The following interactions between the variables were included in the model: ‘Population \* Elevation’, ‘Habitat \* Elevation’ and ‘Population \* Habitat \* Elevation’. Moreover, as the total study period extended over 1 month, the day of the survey (‘Study day’) was included as a random factor. The sample size in the model was 290.

In the two base models for fungal pathogens recorded in non-dis-infected and disinfected seeds, the target variable was the arcsine transformed frequency of true pathogen colonies per petri dish (calcu-lation formula: ‘True pathogens’ = ASIN (SQRT (number of true pa-thogen colonies/number of seeds placed in the petri dish)). Covariates and interactions were similar to the model for leaf damage. However, as the period of seed development is similar irrespective of the study re-gion (only mature seeds were collected), the day of the survey was not included. Because seeds of some plants were placed in two different petri dishes, plant id was added as a random effect (‘Plant ID’). The sample sizes in the models for the non-disinfected and disinfected seeds were 127 and 54, respectively.

It should also be noted that in both models for fungal pathogens

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recorded in seeds, only true pathogens and bacterial colonies were in-cluded, i.e., *Acremonium* sp., *Bipolaris* sp., *Botrytis cinerea*, *Chaetomium* sp., *Fusarium equiseti*, *F*. *oxysporum*, *Fusarium* sp., *Pestalotia* sp., *Phoma* *complanata*, *P. epicoccina*, *P. eupyrena*, *P. exigua*, *P. medicaginis*, *P. neb-ulosa*, *P. pomorum*, *P. sorghina*, *Phoma* sp., *Phytium* sp., *Phytophthora* sp., *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Sordaria fimicola*, *Ulocladium botrytis*, and *Ulocladium chartarum* ([Appendices B–E](#page12)).

Correlation tests between fungal pathogens were conducted with the use of Pearson parametric correlations. The arcsine transformed frequencies of fungal colonies per petri dish were included. All recorded true pathogens and the two most numerous secondary pathogens (*Aureobasidium pullulans* and *Alternaria alternate*) were taken into ac-count. The sample sizes in correlations for the non-disinfected and disinfected seeds were 199 and 137, respectively.

The frequency of flowers visited by particular groups of pollinators at each surveyed locality was also tested. In the base model, the arcsine transformed frequency was the target variable (calculation formula: ‘Frequency of flowers’ = ASIN (SQRT (number of visited flowers/total number of recorded flowers)). The covariates were the insect systematic group (‘Taxon’), surveyed population (‘Population’), habitat type (‘Habitat’), elevation (‘Elevation’) and the variable that represented weather conditions during the survey (‘Weather’); this last variable was obtained from principal component analysis (PCA). This analysis was based on three variables: (1) direct sun radiation or lack of it, (2) average temperature and (3) average wind speed. In PCA, the Kaiser-Meyer-Olkin measure of sampling adequacy was 0.53, with a *p*-value in Bartlett’s test < 0.001. The percentages of variance accounting for the three ob-tained components were 50.5%, 30.0%, and 19.5%. The first component explained most of the variance (with an eigenvalue of 1.516) and was added to the model as the ‘Weather’ variable. The respective component matrix values for that component were 0.82, 0.69 and -0.61. Moreover, in the model, the following interactions were included: ‘Taxon \* Population’, ‘Taxon \* Habitat’, ‘Taxon \* Elevation’ and ‘Taxon \* Population \* Habitat \* Elevation’. Two random effects, ‘Study day’ and recording time (‘Recording time’), were also included in the model – the former effect because the activity of some insects changes over time, and the latter one because in a few cases, the recording was different than 50 min. The sample size in the model was 214.

Pollination time was calculated as a ratio of the total duration of the visit (in seconds) to the number of visited flowers (calculation formula: ‘Pollination time’ = ‘Time of the visit’/‘N visited flowers’). The ob-tained values were rounded up to obtain numerical data, which is ne-cessary in negative binomial regression. In the model, there was only a single covariate, which represents the insect systematic group, ‘Taxon’. The sample size in the model was 126.

In the model for plant size, the target variable were obtained from the PCA. To reduce the dimensions of the data on plant size assessed for each plant, we carried out analysis based on a correlation matrix in-corporating five related variables: (1) number of leaves, (2) average leaf length, (3) average leaf width, (4) stem height and (5) diameter (measured near the ground in centimeters). The Kaiser-Meyer-Olkin measure of sampling adequacy was 0.56, and the *p*-value in Bartlett’s test was < 0.001. Percentages of variance accounting for the five ob-tained components were 49.6%, 33.1%, 12.9%, 3.2% and 1.2%. The two first components explained most of the variance (with eigenvalues of 2.481 and 1.657, respectively) and were added to the analysis as two different size variables (component 1 as ‘Size 1’ and component 2 as ‘Size 2’). The respective component matrix values for component 1 were 0.73, 0.68, 0.64, 0.64 and 0.82, while those for component 2 were -0.52, 0.71, 0.75, -0.33 and -0.46. However, only Size 1 was used as the target variable in the model for plant size because, according to the component matrix values, this variable represents dimensions of plants, whereas Size 2 represents plant growth development strategy. In the model for Size 1, the same covariates, interactions and random effect were used as in the model for leaf damage. The sample size in the model was 288.

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In the model for plant fertility, the target variables were also ob-tained from the PCA. This analysis was based on four variables assessed per plant: (1) number of flowers, (2) pods, (3) mature seeds, and (4) weight of all mature seeds (measured in grams). The Kaiser–Meyer–Olkin measure of sampling adequacy was 0.62, and the *p*-value in Bartlett’s test was < 0.001. The percentages of variance ac-counting for the four obtained components were 56.2%, 24.7%, 15.3% and 3.8%. The first component explained most of the variance (with an eigenvalue of 2.249) and was added to the model as the ‘Fertility’ variable. The respective component matrix values for this component were 0.37, 0.64, 0.92 and 0.92. In the base model for fertility, the same covariates, interactions and random effect were used as in the models for leaf damage and plant size. The sample size in the model was 233.

In the last analysis, performance of seeds was studied using seed weight. In the base model, where average weight of a single seed, re-presentative for the single plant individual, was included as the target variable (‘AVG seed weight’), the same covariates, interactions and random effect were used as in the models for leaf damage, plant size and fertility. The sample size in the model was 233.

The base models were used to generate best-fit models (Appendix F) using the lowest corrected Akaike information (AICc) criterion (Burnham and Anderson, 2002). The only exception was the model for pollination time, with a single covariate and without a random effect, for which it was not possible to generate any better combination of variables.

**3. Results**

*3.1. Species distribution*

*Impatiens balfourii* was found in 73 localities: 12 in Le Rozier, 9 inTorino, 5 in Insubria, 10 in Istria, 10 in Zagreb and 27 in Andorra ([Appendix G](#page12)). In general, the species occurred mainly along streams (*N*

* 20; 27.4%) and roadsides (*N* = 17; 23.3%). However, localities in urban areas (*N* = 9; 12.3%), ruderal areas (*N* = 5; 6.8%), near touristic trails (*N* = 4; 5.5%) and urban green areas (*N* = 4; 5.5%) were also noted. Moreover, in most of the studied regions, the species was culti-vated in gardens (*N* = 14; 19.2%). Distribution of the species in Andorra differed from other areas in that it did not occur along roadsides at all. At the same time, there were 8 records along streams. After excluding those records from the total pool of localities, the species was more often re-corded along roadsides (*N* = 17) than along streams (*N* = 12).

The species preferred moderately shaded localities (average shading

* 55%), with the highest shading at touristic trails (68%) and the lowest at roadsides (45%). At the same time, the average patch area was 5.4 m2. The largest patches were noted in Torino and Insubria (average areas: 13.8 m2 and 9.2 m2; respectively), while the smallest were in Istria and Le Rozier (∼2 m2). Notably, the patch area significantly differed among habitats. By far, the largest patches were recorded along streams and in ruderal areas (10.9 m2 and 9.4 m2; respectively); patches in other habitats were ∼4 times smaller.

*3.2. Enemy pressure on plants*

In total, 46,891 leaves were counted, of which 20.7% (*N* = 9727) had leaf damage (Table 2); each of the surveyed individuals had at least one damaged leaf. The predominant damage symptom in each of the studied populations was ‘rusts and spots’ – 6318 leaves recorded from 281 plants (96.9% of all studied individuals; Table 2). Necrosis was the least frequent symptom, recorded only from 29 leaves of 19 plants (6.5% of all studied individuals; Table 2). Prevalence of the symptoms differed between the populations. For instance, in Istria, discoloration was the second dominant symptom, while in Zagreb, it played only a minor role ([Appendix A](#page12)).

There were significant differences in the level of enemy pressure – expressed as the level of leaf damage – between the surveyed

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**Table 2**

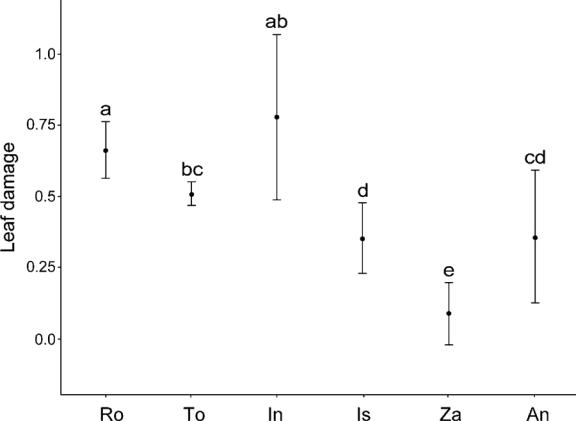
Total number of *Impatiens balfourii* individuals with leaf damage and number of damaged leaves.

|  |  |  |
| --- | --- | --- |
| Symptom | *N* plants with leaf damage | *N* damaged leaves |
|  |  |  |
| All symptoms | 290 | 9727 |
| Rusts and spots | 281 | 6318 |
| Deformations | 148 | 1598 |
| Browsings | 148 | 819 |
| Discolorations | 99 | 841 |
| Mines | 43 | 122 |
| Necrosis | 19 | 29 |
|  |  |  |

**Table 3**

GLMM model for leaf damage. The model compares individuals of *Impatiens* *balfourii* from populations differing in age. Habitat type and elevation were alsoincluded in the model.

|  |  |  |  |
| --- | --- | --- | --- |
| Effect | *F* | df | *p* |
|  |  |  |  |
| Population | 10.95 | 279 | < 0.001 |
| Habitat | 29.68 | 278 | < 0.001 |
| Elevation | 4.92 | 275 | 0.027 |
| Population \* Elevation | 12.83 | 279 | < 0.001 |
|  |  |  |  |



**Fig. 2.** Estimated mean frequency of leaf damage ( ± SE) recorded on*Impatiens**balfourii* from older (Ro – Le Rozier, To – Torino, In – Insubria) and younger (Is

– Istria, Za – Zagreb, An – Andorra) populations. Dissimilar letters above the error bars indicate significant differences between the populations.

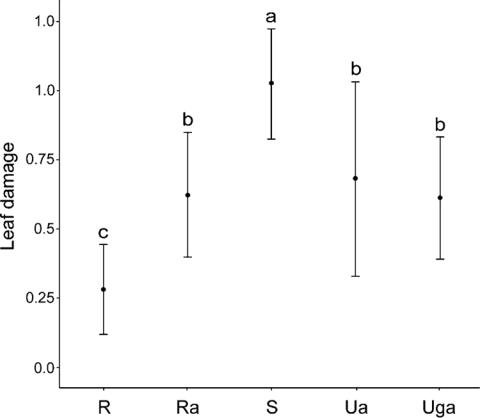
individuals from distinct populations and habitats (Table 3). In almost all cases, the older populations were under a higher enemy pressure than the younger ones (contrasts: *p* < 0.03; Fig. 2). The exception was the comparison between Andorra and Torino, for which the differences were statistically non-significant. However, it should be noted that in this comparison, there is also a tendency that younger population seems to be less affected by enemies than older one ([Fig. 2](#page6)).

The enemy pressure was lowest at roadsides and highest at streamsides (contrasts: *p* < 0.005 in all cases; Fig. 3). Plants recorded from ruderal, urban and urban green areas did not differ in terms of the enemy release potential. However, the species was recorded in these habitats less often than along roadsides and streamsides (Table 1). It should also be noted that in 5 of the 6 surveyed populations, the fre-quency of recorded damage decreased with elevation (*F*1,274 = 4.92, *p* = 0.027); the exception was Istria, where the trend was opposite.

*3.3. Enemy pressure on seeds*

The number and frequency of colonies of pathogenic fungi recorded on disinfected and non-disinfected seeds are included in Appendices B–E.

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**Fig. 3.** Estimated mean frequency of leaf damage ( ± SE) recorded on*Impatiens**balfourii* from different habitats (R – roadside, Ra – ruderal area, S – streamside,Ua – urban area, Uga – urban green area). Dissimilar letters above the error bars indicate significant differences between the habitats.

**Table 4**

GLMM model for true pathogens recorded on non-disinfected and disinfected seeds of *Impatiens balfourii*. The model compares seeds collected from popula-tions differing in age. Habitat type and elevation were also included in the model.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Disinfection | Effect | *F* | df | *p* |  |
|  |  |  |  |  |  |
| Non-disinfected seeds | Population | 3.23 | 123 | 0.009 |  |
| Disinfected seeds | Habitat | 1.58 | 121 | > 0.1 |  |
| Population | 1.32 | 50 | > 0.2 |  |
|  | Habitat | 2.10 | 48 | > 0.1 |  |
|  |  |  |  |  |  |

In the model for non-disinfected seeds (Table 4), most of the com-parisons between populations were non-significant (Fig. 4ND). Only in two contrast comparisons were seeds from the older populations under a higher pathogen pressure than those from the younger ones: Insubria vs Zagreb (contrasts: SE = 0.076, *t* = 1.986, df = 118, *p* = 0.049; [Fig. 4](#page7)ND) and Insubria vs Andorra (contrasts: SE = 0.100, *t* = 3.104, df

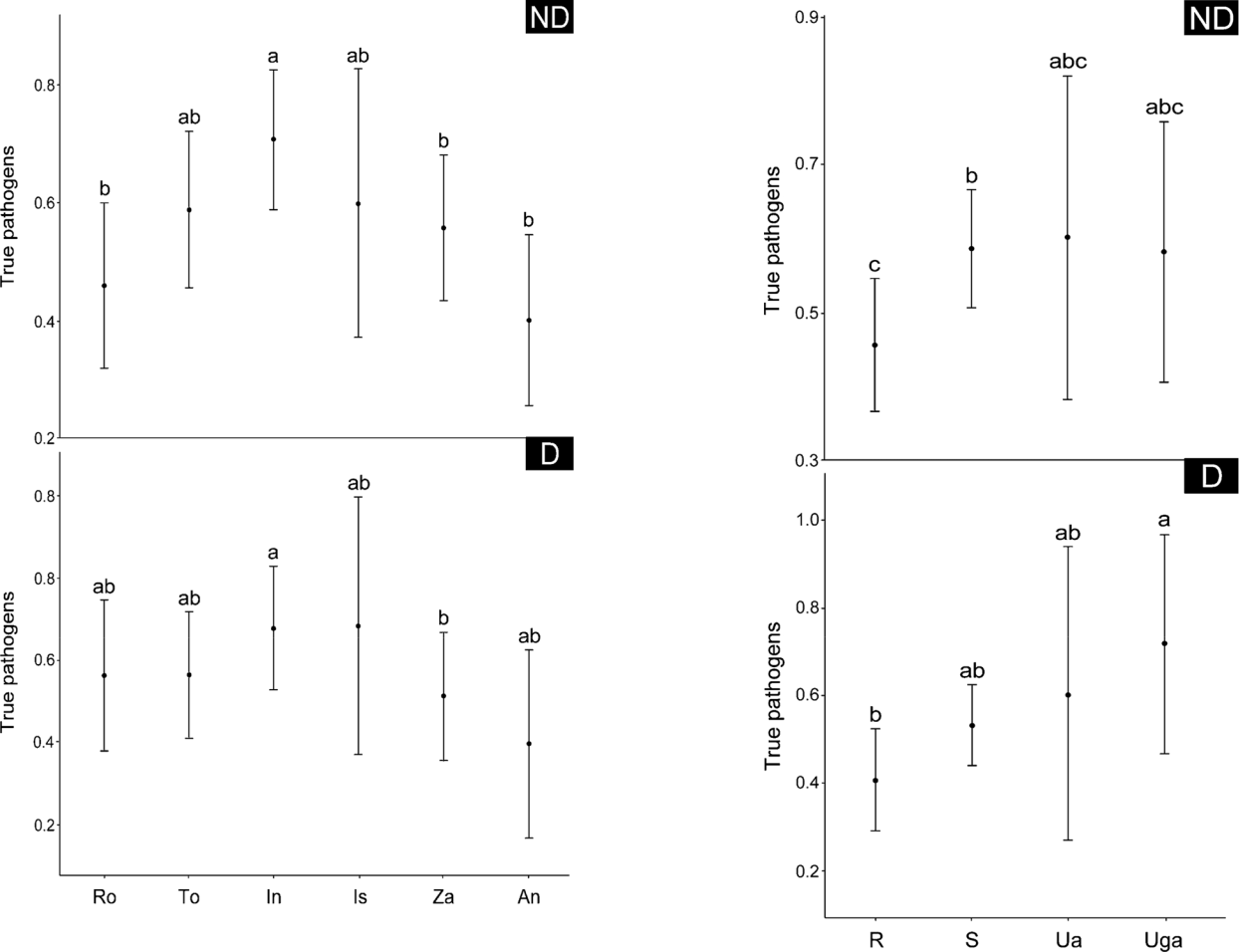
* 118, *p* = 0.002; [Fig. 4](#page7)ND). Nevertheless, Zagreb and Andorra did not differ from the two other older populations – Torino and Le Rozier ([Fig. 4](#page7)ND). The general result for enemy pressure in different habitats was non-significant for non-disinfected seeds ([Table 4](#page6)); however, the contrasts revealed differences between roadsides and streamsides (contrasts: SE = 0.061, *t* = −2.120, df = 118, *p* = 0.036). As in the model for leaf damage, the enemy pressure on seeds of plants occurring along streams was higher than that on those from roadsides ([Fig. 5](#page7)ND). There were no differences between other habitats. Ruderal areas were not included in the analysis because there were no records of true pa-thogens there.

In the model for disinfected seeds, the overall result was non-sig-nificant ([Table 4](#page6)). The only significant difference found in contrast comparisons was that the frequency of true pathogen colonies recorded from the Insubrian seeds was higher than the one found in the Zagreb population (contrasts: SE = 0.081, *t* = 2.047, df = 45, *p* = 0.046; [Fig. 4](#page7)D). However, as in the model for non-disinfected seeds, the result for this younger population did not differ from the results obtained for the older Torino and Le Rosier populations ([Fig. 4](#page7)D). Similarly, in case of disinfected seeds, there was only one significant result for habitat type, with the pathogen pressure on seeds from urban green areas higher than that on those from roadsides (contrasts: SE = 0.136, *t* = 2.290, df = 45, *p* = 0.027; [Fig. 5](#page7)D). However, as in the earlier model, the average value was lowest for roadsides ([Fig. 5](#page7)), and ruderal areas were not included.

The most numerous fungal species on both disinfected and non-disinfected seeds was a secondary pathogen, *Aureobasidium pullulans*

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**Fig. 4.** Estimated mean frequency of true pathogen colonies per petri dish( ± SE) recorded on non-disinfected (ND) and disinfected seeds (D) of *Impatiens* *balfourii* from older (Ro – Le Rozier, To – Torino, In – Insubria) and younger (Is

– Istria, Za – Zagreb, An – Andorra) populations. Dissimilar letters above the error bars indicate significant differences between the populations.

(Appendices B–E). In disinfected seeds, the frequency of *A. pullulans* colonies correlated negatively with the combined frequency of all co-lonies of true pathogen species (*r*S = −0.171, *p* = 0.045).

Moreover, we found that in Zagreb – where the frequency of leaf damage was lowest – *A. pullulans* was recorded from seeds significantly more often than in the five remaining populations (Appendices B–E). Similarly, it was the predominant fungus at roadsides, where leaf da-mage level was lowest among all habitats (Appendices B–E). This trend was demonstrated for both non-disinfected and disinfected seeds.

Unexpectedly, we also found that the number of colonies of *A. pull-ulans* on disinfected seeds correlated negatively with the frequency ofcolonies of *Alternaria alternate* (*r*S = −0.167, *p* = 0.051) – a species known to reduce a true pathogen, *Fusarium culmorum* (Liggitt et al., 1997).

*3.4. Pollinator activity*

In general, bumblebees were the predominant group (*N* = 110,

51.4%) of pollinators. *Syrphidae* and *Apidae* were also abundant (*N* = 47, 22%; *N* = 40, 18%, respectively), while *Sphingidae* and *Diptera* were recorded rarely (*N* = 10, 47%; *N* = 6, 2.8%; respectively). Only one specimen of *Vespidae* was recorded (0.5%). Moreover, a few pollinators were identified to the species level: *Bombus terrestris* (Torino, Insubria, Istria, Zagreb, Andorra), *B. pascuorum* (Istria), and *Macroglossum stel-latarum* (Le Rozier, Torino, Istria, Andorra). We also recorded bum-blebees, which are classified as *B. lucorum* complex (Insubria, Istria, Torino, Zagreb).

**Fig. 5.** Estimated mean frequency of true pathogen colonies per petri dish( ± SE) recorded on non-disinfected (plot ND) and disinfected (plot D) seeds of *Impatiens balfourii* from different habitats (R – roadside, S – streamside, Ua –urban area, Uga – urban green area). Dissimilar letters above the error bars indicate significant differences between the populations.

Pollinator activity differed between the populations, with the Zagreb plants being visited most frequently (contrasts: *p* < 0.002 in all cases; Table 5; Fig. 6). Interestingly, in this young population, the fre-quency of recorded leaf damage was lowest, and the secondary pa-thogen *A. pullulans* was most abundant here. The frequency of visits was also relatively high for Le Rozier (contrasts: *p* < 0.02 in all cases except for comparison between Le Rozier and Insubria, with *p* = 0.2; Fig. 6), where the total number of damaged leaves was very high (Fig. 2). Moreover, in this population, ‘rusts and spots’ and ‘browsings’ were the most recorded leaf damage types ([Appendix A](#page12)).

Frequency of visited flowers was highest for both urban green areas and ruderal areas (contrasts: *p* < 0.02 in all cases except for comparison between ruderal areas and streams, with *p* = 0.07; Fig. 7), whereas the lowest frequency was in urban areas (contrasts: *p* < 0.03 in all cases; Fig. 7).

**Table 5**

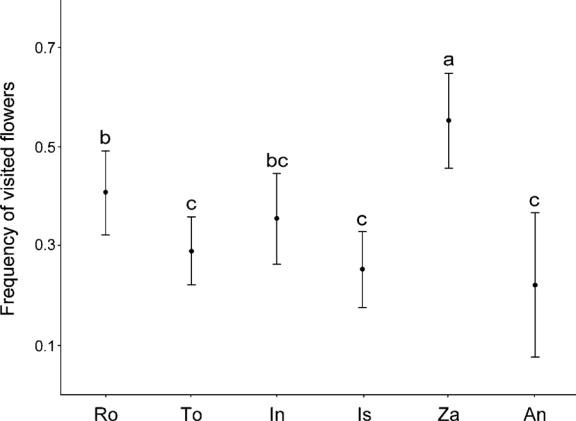
GLMM model for frequency of flowers visited by pollinators. The model com-pares individuals of *Impatiens balfourii* from populations differing in age. Habitat type, elevation and taxon were also included in the model.

|  |  |  |  |
| --- | --- | --- | --- |
| Effect | *F* | df | *p* |
|  |  |  |  |
| Population | 11.632 | 203 | < 0.001 |
| Habitat | 6.914 | 202 | < 0.001 |
| Taxon × elevation | 4.233 | 204 | < 0.001 |
|  |  |  |  |

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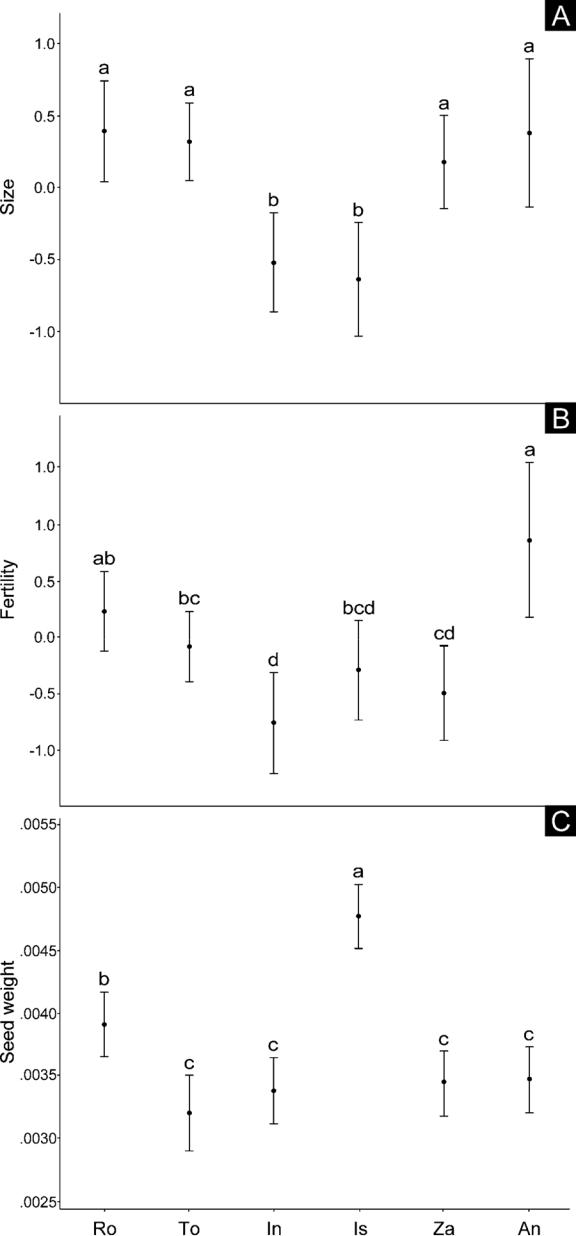
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**Table 6**

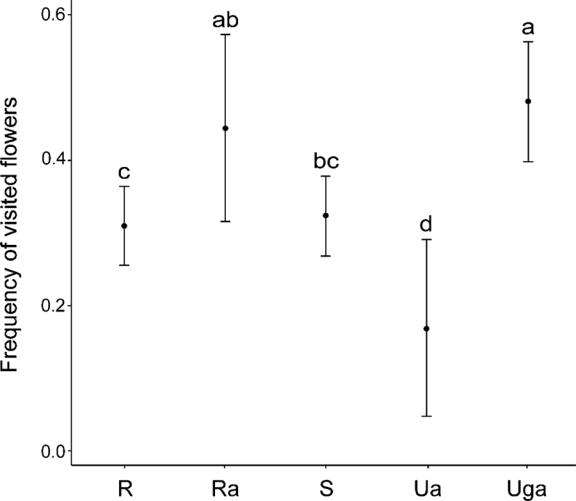


GLMM models for ‘Size 1′, ‘Fertility’ and ‘AVG seed weight’ target variables. The models compare individuals of *Impatiens balfourii* from populations differing in age. Habitat type and elevation were also included in the models.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Target variable | Effect | *F* | df | *p* |  |
|  |  |  |  |  |  |
| Size 1 | Population | 10.32 | 282 | < 0.001 |  |
|  | Habitat | 24.57 | 281 | < 0.001 |  |
| Fertility | Elevation | 6.01 | 278 | 0.014 |  |
| Population | 5.49 | 227 | < 0.001 |  |
|  | Habitat | 7.07 | 226 | < 0.001 |  |
| AVG seed weight | Elevation | 1.28 | 223 | > 0.2 |  |
| Population | 19.52 | 232 | < 0.001 |  |
|  | Habitat | 12.72 | 227 | < 0.001 |  |
|  |  |  |  |  |  |



**Fig. 6.** Estimated mean frequency of flowers of*Impatiens balfourii*visited bypollinators ( ± SE) from older (Ro – Le Rozier, To – Torino, In – Insubria) and younger (Is – Istria, Za – Zagreb, An – Andorra) populations. Dissimilar letters above the error bars indicate significant differences between the populations.



**Fig. 7.** Estimated mean frequency of flowers of*Impatiens balfourii*visited bypollinators ( ± SE) from different habitats (R – roadside, Ra – ruderal area, S – streamside, Ua – urban area, Uga – urban green area). Dissimilar letters above the error bars indicate significant differences between the habitats.

We also found that the activity of pollinators decreased with ele-vation (interaction between taxon and elevation, Table 5). This result was particularly pronounced for *Apidae,* and there was only one ex-ception – the number of records of *Sphingidae* increased with altitude. However, in general, most pollinators were recorded between 131 and 382 m a.s.l. (increasing from Zagreb, through Insubria and Torino to Le Rozier), with a decrease between 481 and 666 m a.s.l. (Le Rozier, Torino, Istria), and particularly between 856 and 1092 m a.s.l. (An-dorra).

The recorded groups of insects differed in pollination time (*F*4, 121 = 7.302, *p* < 0.001; Appendix H). The shortest average pollination time was recorded for *Sphingidae* (2.33 s) and *Bombini* (4.73 s).

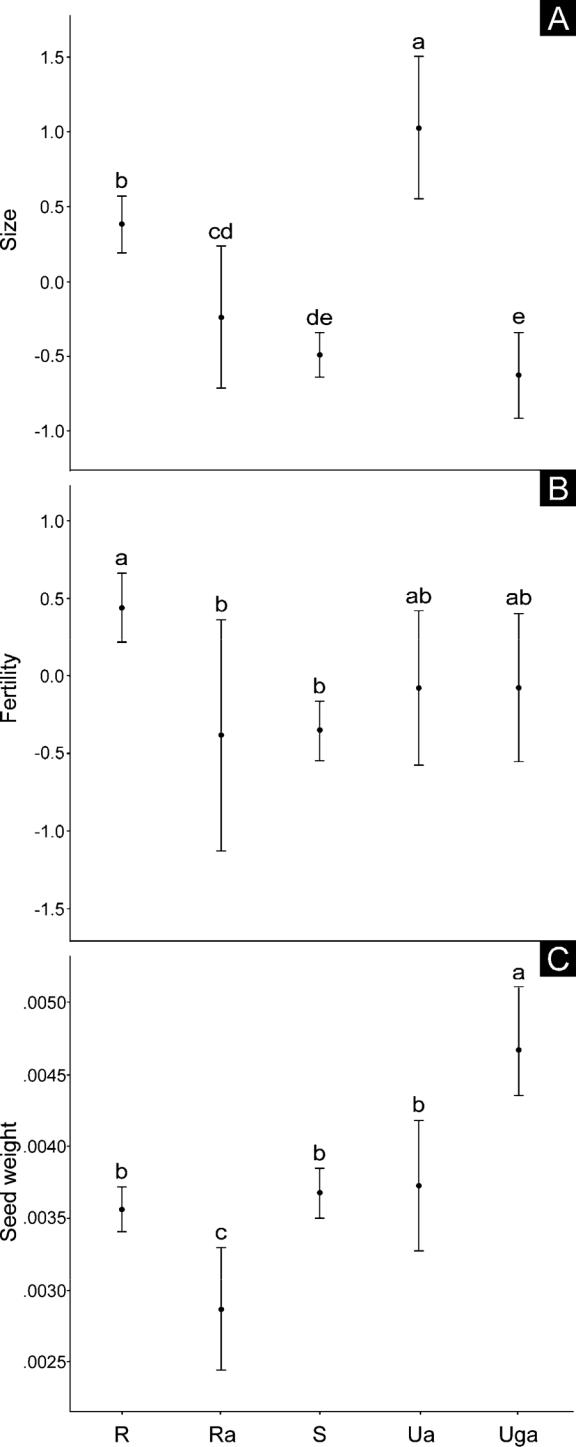
*3.5. Assessment of plant performance and seed quality*

In general, the size of the plants decreased with altitude (*F*1, 277 = 6.01, *p* = 0.014; Table 6), and this trend was not observed only in Istria and Zagreb. Moreover, there were also significant differences between the populations; individuals from Insubria and Istria were significantly smaller than those from the four other populations (contrasts: *p* < 0.007 in all cases;Fig. 8A). Considering habitats, the largest

**Fig. 8.** Estimated mean plant size ( ± SE; plot A), mean fertility ( ± SE; plot B)and mean seed weight ( ± SE; plot C) of *Impatiens balfourii* individuals and seeds from older (Ro – Le Rozier, To – Torino, In – Insubria) and younger (Is – Istria, Za – Zagreb, An – Andorra) populations. Dissimilar letters above the error bars indicate significant differences between the populations.

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**Fig. 9.** Estimated mean plant size ( ± SE; plot A), mean fertility ( ± SE; plot B)and mean seed weight ( ± SE; plot C) of *Impatiens balfourii* individuals and seeds from different habitats (R – roadside, Ra – ruderal area, S – streamside, Ua

– urban area, Uga – urban green area). Dissimilar letters above the error bars indicate significant differences between the habitats.

individuals were noted from urban areas (contrasts: *p* < 0.01 in all

cases; Fig. 9A) and roadsides (contrasts: *p* < 0.01 in all cases), whereas the smallest plants grew along streams and in urban green areas (con-trasts: *p* < 0.001 in all cases). The size of the plants from ruderal areas was intermediate (contrasts: *p* < 0.01 in all cases; Fig. 9A). Moreover, the results of the Size 2 variable – obtained in the PCA (see component 2 matrix values in Methods) – showed that the length and width of

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leaves increased with decreases in three other variables (N of leaves, stem height and diameter).

The results of the model for ‘Fertility’ showed that individuals from Andorra and Le Rozier had the highest reproductive potential (Fig. 8B). However, it should be stressed that although the plants from these two study regions did not differ statistically (contrast: SE = 0.42, t = -1.51, df = 222, *p* > 0.1), only the Andorran individuals were more fertile than plants from the four other populations (contrasts: *p* < 0.01 in all cases; Fig. 8B). Plants from Le Rozier were more fertile only in com-parison to Insubria and Zagreb (contrasts: *p* < 0.001 in all cases; Fig. 8B), where the fertility levels were very low. Differences between the surveyed habitats were less pronounced than those between the populations (Fig. 9B); however, roadsides obviously dominated in comparison to ruderal areas and streams (contrasts: *p* < 0.035 in all cases), and fertility in this habitat was slightly higher than that in urban and urban green areas ([Fig. 9](#page9)B).

Seeds from Istria were heaviest (contrasts: *p* < 0.001 in all cases; Fig. 8C). Seeds from Le Rozier were lighter than those from Istria; however, they dominated over the four remaining populations (con-trasts: *p* < 0.006 in all cases; Fig. 8C). There were no differences in seed weight between Torino, Insubria, Zagreb and Andorra (Fig. 8C). As far as habitats are concerned, seeds in urban green areas were heaviest (contrasts: *p* < 0.002 in all cases; Fig. 9C), followed by seeds from roadsides, streamsides and urban areas (contrasts: *p* < 0.01 in all cases; Fig. 9C). The lightest seeds were produced by plants that occurred in ruderal areas (contrasts: *p* < 0.01 in all cases; Fig. 9C).

**4. Discussion**

*4.1. Enemy pressure on plants and assessment of their performance*

In general, the leaf damage tests showed that the older populations of *I. balfourii* were under a higher enemy pressure than the younger ones. However, we found only a relatively weak support for the EICA assumption that in the absence of enemies, resources saved on defence are re-allocated into increasing growth or reproduction (Blossey and Notzold, 1995). Such results are similar to those obtained in the EICA study of *I. glandulifera* (Gruntman et al., 2017): although high enemy release abilities in younger populations of that species were confirmed, this release had no effect on increasing its allelopathic potential. Therefore, neither the results for *I. balfourii* nor those for *I. glandulifera* are fully consistent with the EICA hypothesis.

Tests of plant size demonstrated that elevation plays an important and complex role for the species, regardless of the study region. In al-most all populations, the frequency of the recorded leaf damage and the plant size decreased with elevation. Therefore, resources saved on the defence against enemies at higher elevations were not re-allocated into a larger plant size. At the same time, it should be noted that in all the study regions, *I. balfourii* occurred significantly more often at higher elevations than *I. glandulifera*. In the Pyrenees, the highest elevation for the former species was 1124 m a.s.l. (Les Escaldes, Andorra), while for the latter, the highest elevation was only 575 m a.s.l. (Lassur, France). Thus, it cannot be excluded that at the invasion forefront, *I. balfourii* re-allocates its resources saved thanks to release from the enemies into traits that allow its further vertical spread into montane areas. In harsh climate conditions, traits such as higher frost resistance and sufficient fertility may be the most important prerequisites for survival.

Despite this general trend for leaf damage, the results from Istria were ambiguous: here, the level of leaf damage increased with the elevation. This result stands contrary to the commonly acknowledged phenomenon of the abundance and diversity of organisms (hence also enemies) decreasing with elevation (Najberek et al., 2019; [Rahbek,](#page13) [1995](#page13)). However, such ambiguous results for *Impatiens* were also re-corded in other European regions, e.g., in the Polish mountains (Najberek et al., 2016). They can be governed by local environmental conditions, such as temperature, precipitation, oxygen availability and

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wind turbulence, additionally complicated by differences in the ways in which different organisms respond to fluctuations in these variables. It cannot be excluded that the particularly dry Istrian climate, with its permanent water deficits and heat stress (Karleuša et al., 2018), may be more lethal for the pathogens at lower elevations than up in montane areas.

Ambiguous results were noted also for Insubria, where the plants were smallest although the altitudes are low and the mild local climate favors thermophilous species (Najberek et al., 2017). A possible ex-planation for the small size of *I. balfourii* in this area is that until re-cently, the population was widespread and distributed mainly (81% of sites) along roads (Najberek et al., 2017). As we argue, while roadsides are preferred by the species, it is possible that they may in fact act as an ecological trap because of mowing. This can be illustrated by the fact that the roads studied in 2016 (Najberek et al., 2017) were re-constructed in 2018, which resulted in reducing the number of localities of *I. balfourii* along 3 out of 4 the studied transects, from a total of 49 to only 3. The small size of the plants recorded in 2018 may therefore be a consequence of the fact that the only individuals who survived the road maintenance practices were those that germinated very late in the season (after roadside mowing), whereas typically germinating plants had been eradicated. It is known that late germination may negatively impact plant size (Gioria et al., 2016). It should also be noted that the enemy pressure on smaller populations is stronger than on larger ones (Steffan-Dewenter and Schiele, 2008); thus, it cannot be excluded that the high level of leaf damage revealed in Insubria could also be a result of road maintenance practices that regularly decimated the local po-pulation.

In general, as in the case of plant size, the results of fertility tests did not comply with the results of the enemy release tests for leaf damage. However, a noteworthy exception was Andorra: while its population was youngest of all that we studied, its fertility was high. According to the EICA hypothesis assumptions, high fertility was related to the low level of leaf damage recorded in this region. The enemy release of the Andorran plants was sharply pronounced in comparison with the plants from Insubria and Le Rozier; however, also in Torino, the plants seemed to be under a higher enemy attack than in Andorra. At the same time, in all studied populations, pollinator activity (with the exception of *Sphingidae*) decreased with elevation and was lowest in Andorra. Thus,plants in this region may be forced to use autonomous self-pollination without strictly relying on external pollen vectors (*Apidae*, *Diptera* and *Vespidae* were not recorded in Andorra). *I. balfourii* is self-compatible,which does not lead to inbreeding depression (Jacquemart et al., 2015), and as Baker’s law suggests, self-compatible species spread more quickly than self-incompatible ones (Baker, 1955; Pyšek et al., 2011). Therefore, this strategy may promote invasion of *I. balfourii* in regions where activity of pollinators is insufficient, which could be the case in Andorra. It should also be noted that in this region, *Bombini* and *Sphingidae* were present, and those groups had the shortest pollinationtime. Both plants and pollinators may benefit from a short pollination time (Leonard et al., 2011); therefore, it could be assumed that the although the pollinator groups were limited to *Bombini* and *Sphingidae* in Andorra, these insects pollinate *I. balfourii* in an effective way. Moreover, we found that the pollination activity of the latter group increased with elevation. This result seems to strongly indicate that in Andorra, *I. balfourii* was not entirely deprived of pollination service.

Interestingly, a relatively high fertility was found in plants from Le Rozier. In this older population, the level of enemy pressure was high; thus, the potential to save resources on defence was limited. This result was contrary to the EICA hypothesis, and it is likely that other factors were at play in this region, such as insufficient resources provided by other flowering plants for pollinating insects there. If the diversity or blossom density of other food plants was low, it could enhance polli-nator activity to *I. balfourii*. It is known that pollination improves plant fertilization (Ollerton et al., 2011). Ambiguous results were noted also for Istria and Insubria. The low fertility in Istria was probably

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determined by extreme water stress, characteristic for this area. Water stress increases flower abortion and results in low numbers of devel-oped flowers (Descamps et al., 2018). The drivers of low fertility of plants in Insubria were probably similar to factors responsible for their small size, which could be a result of late germination of maternal plants that had survived road construction works.

It cannot be excluded that, due to the extensive geographic scale of the study, in some populations, the timing of the surveys did not match the peak seed setting period. The possible disparity in this respect was accounted for by including the day of survey into the models. However, it is possible that the date-induced bias was not fully compensated for; in the Zagrebian population, which was surveyed earliest and in which the EICA was not confirmed, the recorded fertility was lowest despite low enemy pressure and high pollinator activity.

*4.2. Enemy pressure on seeds and assessment of their quality*

Most tests revealed that the pressure exerted by the enemies on seeds was fairly constant with EICA hypothesis in all the studied po-pulations. The only significant differences concordant with EICA as-sumptions were detected between the older Insubrian and two younger populations in Zagreb and Andorra from non-disinfected seeds, while in disinfected seeds, the difference held only between Insubria and Zagreb. Nevertheless, these results provide weak support for the EICA assumptions because in both non-disinfected and disinfected seeds, the younger Zagreb and Andorra did not differ from the older Le Rozier and Torino. Since the evolution of the ability to release from enemies al-ready as early as the seed stage would be beneficial to plants, parti-cularly to annuals, which disperse only by seeds, high enemy pressure on seeds could in fact be the factor limiting the spread of *I. balfourii*. It is especially pronounced in the tests of disinfected seeds, which demon-strated the resistance of the seed core against pathogens. In our earlier study, we demonstrated that seeds of *I. glandulifera* and *I. balfourii* from Insubria could prevent true pathogens from penetrating the seed core, which may be associated with antimicrobial, antioxidant, and/or alle-lopathic abilities of this genus (Najberek et al., 2018). The EICA as-sumptions cannot be validated by comparison with the invasive *I.* *glandulifera* or other invasive alien plants, as there have been no studiesto date on enemy pressure on seeds from populations differing in age. It should also be stressed that although the capability of seeds to save resources and invest them into development of any trait may not seem straightforward, a lower enemy pressure on seeds may in fact be di-rectly translated into better conditions of seedlings (Najberek et al., [2018](#page13)). Thus, individual species may evolve their competitive abilities in different ways, including allocating resources at different development stages.

It is known that the seed size strongly determines the fitness, growth and survival potential of adult plants (Čuda et al., 2016; [Olejniczak](#page13) et al., 2018). Since large seeds provide more resources for seedlings, in our studies, we used the weight of seeds from each population as a proxy for the seed condition. However, the obtained results provided no support for the EICA assumptions. Only the Istrian plants, with the heaviest seeds, stood out in this respect from all the other populations. Those plants grew in a particularly severe climate, with water deficit and heat stress (Karleuša et al., 2018); thus, the high quality of seeds was probably a necessary pre-requisite to ensure the survival of seed-lings. Nevertheless, the seed weight was not reflected in the results of the tests of enemy release in seeds, which indicated a lack of differences between Istria and other populations in terms of enemy pressure; the outcome might indicate that the Istrian population is capable of better maintaining fitness of its seeds despite unfavourable environments, which is directly connected with high phenotypic plasticity of the Is-trian *I. balfourii* individuals ([Richards et al., 2006](#page13)).

Another interesting result of testing the enemy release of seeds was that the secondary pathogen *Aureobasidium pullulans* was the most abundant fungus; this species produces antibacterial and antifungal

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compounds, which allows the host plant to increase resistance against enemies (Bozoudi and Tsaltas, 2018). We showed that this pathogen may reduce the number of true pathogens (e.g., *Fusarium* species) that are very harmful for seeds (Najberek et al., 2018). Moreover, the high abundance of *A. pullulans* may have contributed to the lowest level of leaf damage in Zagreb, where the number of colonies of the pathogen was significantly higher than in the other populations. We suppose that *A. pullulans* also occurred on *I. balfourii* leaves; however, it was notdirectly tested. We also found that the occurrence of *A. pullulans* cor-related negatively with *Alternaria alternate* – another secondary pa-thogen whose antifungal activity was found to limit the true pathogen *F. culmorum* (Liggitt et al., 1997). Such antagonistic associations be-tween *A. pullulans* and *A. alternate* may apparently weaken their sy-nergistic beneficial impact on limiting true enemies. From the EICA perspective, however, it can be assumed that the presence of one of these species could positively influence seed resistance against enemies, resulting in increasing their competitive abilities.

*4.3. Maladaptive habitat preferences*

The result of the tests of habitat preferences was that individuals of *I. balfourii* from roadsides released from their enemies better than thosegrowing in ruderal areas, streamsides, urban areas or urban green areas. The difference was not driven by the study region; thus, it was de-termined by environmental conditions rather than by evolutionary shifts. However, as we did not perform genetic or common garden tests, this conclusion needs to be treated with due caution. Both processes might just as well have played some role. Notably, the result was fully confirmed in the tests of leaf damage for adult plants, and partly for seeds. In consequence, it is probable that the occurrence of *I. balfourii* along roadsides is influenced by the lower pressure of enemies there.

In both previous (Najberek et al., 2017) and present studies, we demonstrated that, although preferred by *I. balfourii*, roadsides may in fact be an ecological trap for the species. Interestingly, the species was not found along roadsides in Andorra. At the same time, the number of the localities recorded in Andorra was highest, but they were situated along streams and in built-up areas of montane towns. Such a pattern may indicate that plants from this young population may have sig-nificantly evolved traits favoring occurrence along streams. In terms of human activity, moist habitats are less frequently disturbed and therefore may be “safer” for plants than roadsides. Other advantages include high propagule pressure, reduced competition from native species, and the wide range of available microhabitats along water courses (Čuda et al., 2017; Hufbauer et al., 2012; Planty-Tabacchi et al., [1996](#page13); Stromberg et al., 2007). It is also known that *I. balfourii* has been evolving floating ability of its seeds (Najberek et al., 2020); however, it was confirmed only for seeds from Istria and Insubria. Nevertheless, it is very likely that seed floating ability in Andorra may have evolved in the same way.

The analyses of plant performance data pooled for all habitats, in-stead of populations, revealed that plants from roadsides (and from urban areas) were larger than those from the remaining habitats. This size difference may indicate that roadside plants, growing under the lowest enemy pressure, can invest saved resources into their growth. Additionally, the large size of plants from urban areas and roadsides may be determined by soil attributes. The substrate in those habitats may be rich in sand that is transported during road and pavement construction and maintenance. Mixed with native soil, that sand pro-vides conditions suitable for plant growth, as was confirmed in a common garden experiment (Najberek et al., 2020). Moreover, the differences between the size of seeds from roadsides, streamsides and ruderal areas that we detected at the European scale were similar to those found in our earlier studies on the Swiss-Italian border ([Najberek](#page13) [et al., 2017](#page13)).

Similar to the results obtained for plant size, analyses of the fertility of plants growing in different habitats, irrespective of the studied

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population, also provided partial support for the conclusion that roadside plants may invest the saved resources into their performance. The plants from roadsides had relatively high reproductive potential. Moreover, the frequency of flowers visited by pollinators was high in this habitat. Only ruderal areas and urban green areas were better in this respect, although it should be noted that high pollinator activity in these two habitats may be a consequence of the proximity of cultivated ornamental vegetation that is rich in flowers (e.g., in city gardens and parks).

We found that seeds from urban green areas were heaviest; hence, assuming a positive relationship between seed size and plant fitness (Čuda et al., 2016; Olejniczak et al., 2018), the quality of seeds in that habitat was highest. In addition to the enemy release tests as well as pollinator activity and fertility assessments, this result indicates that this habitat is suitable for *I. balfourii*. This finding is in agreement with the results suggesting that urban green areas may provide favorable conditions for pollinators thanks to the density, variety, and continuity of flowers occurring there (Goulson et al., 2008). However, urban green areas are unlikely to play an important role in the long-distance spread of the species due to limited connectivity between patches of this ha-bitat. In contrast, linear structures, such as streams and roads, are known to provide suitable invasion corridors (Benedetti and Morelli, [2017](#page12); Čuda et al., 2017). As the quality of seeds produced by *I. balfourii* in these types of habitats was high, it can be presumed that they play a more important role for the large-scale spread of this species.

**5. Conclusions**

The results of the enemy release tests conducted for adult plants show that the level of enemy pressure on the non-invasive *I. balfourii* was lowest in younger populations. However, plants evolved to re-allocate resources previously spent on defence against enemies in only the youngest population, at the invasion front in Andorra. Both low level of leaf damage and increasing fertility were recorded there. Thus, according to the EICA assumptions, only Andorran plants may be competitively superior over native species. At the same time, at the seed stage, highly important for plants dispersing only by seeds, there were no results supporting the EICA assumptions. Thus, the overall result of our study provides relatively weak support for the EICA hypothesis.

We also demonstrated that, except for Andorra, the species prefers maladaptive roadsides. This preference may be a consequence of the lower pressure of enemies in that habitat. At the same time, we showed that plants from roadsides can invest the saved resources into compe-titive ability, which was revealed in all studied populations where the species occurred along roadsides. However, systematically mowed roadsides constitute an ecological trap for the species. Therefore, it cannot be excluded that *I. balfourii* may fall into an ecological trap in European regions with a strict roadside maintenance regime, although a large-scale assessment of the impact of roadside mowing on the popu-lation dynamics is difficult. The importance of this practice can only be illustrated by the fact that within the three months of field surveys on the Swiss-Italian border (Najberek et al., 2017; present study), the po-pulations of *I. balfourii* were regularly decimated. Further studies in this respect are needed.

The presented results suggest that for most of the studied popula-tions, the immediate risk of fast invasion is rather low. The exception may be Andorra and surrounding areas, where the species was abun-dant along streams and completely absent from roadsides. Streamsides are safer than roadsides in terms of human disturbance and provide suitable conditions to disperse and establish new populations ([Benedetti](#page12) and Morelli, 2017; Čuda et al., 2017), which may outweigh the benefits of high enemy release along roads. Interestingly, in all studied popu-lations, patches of the species were largest along streams. It cannot be excluded, therefore, that unless targeted control measures are under-taken, streams may play a crucial role in the future invasion of *I. bal-fourii* in the Pyrenees.

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Another contributing factor when considering possible future sce-narios of *I. balfourii* invasion in the Pyrenees and other European re-gions is that, according to the EU legislation (European Commission, [2017](#page12)), as of 2017, the trade in *I. glandulifera* is banned in response to the threats it poses. There are earlier examples (Scalera, 2007) in which the exclusion of an invasive alien species from trade prompted the in-crease in trade of similar replacement species that have become equally invasive. The similarities of the ornamental values of *I. balfourii* make it an obvious candidate to replace its banned invasive counterpart in trade. The increases in popularity in trade and cultivation would in-evitably lead to an increase in propagule pressure, which could be crucial for increasing the rate of invasion of *I. balfourii* on a large scale.

Moreover, taking into account the similarity between *I. balfourii* and *I. glandulifera* (Najberek et al., 2018) and that the former species wasintroduced approximately 60 years after latter one (Adamowski, 2009), the repeated scenario of increasing the competitive ability of *I. balfourii* is possible. It may suggest that the invasiveness of this species may increase in the near future. The importance of self-compatibility and the fact that it is pollinated by a variety of insects (Pyšek et al., 2011) may also be contributing factors for this scenario.

**Author contributions**

**KN:** original research idea, study design, funding acquisition, pro-ject administration, field surveys and experiments, statistical analyses, writing and editing of the original draft; **WS:** study design, reviewing and editing the draft; **WP:** mycological analyses, reviewing and editing the draft; **KP:** mycological analyses, reviewing and editing the draft; **PO:** study design, statistical analyses, reviewing and editing the draft.

**Funding**

This work was supported by the National Science Centre in Poland under Grant number 2018/02/X/NZ9/00165.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influ-ence the work reported in this paper.

**Acknowledgments**

We would like to thank Guillaume Fried (French Agency for Food, Environmental and Occupational Health & Safety), Frantz Hopkins (Parc National des Cévennes, France), Jérôme Dao (Conservatoire Botanique National des Pyrénées et de Midi-Pyrénées, France) and Slavko Brana (Natura Histrica, Croatia) for their help in determining the dates of the earliest introductions of *Impatiens balfourii* into Le Rozier, Andorra, Istria and Zagreb. We would also like to thank Agata Kaczmarek for her help in mycological analyses.

**Appendices A–H**

Supplementary material related to this article can be found, in the online version, at doi:[https://doi.org/10.1016/j.envexpbot.2020.](https://doi.org/10.1016/j.envexpbot.2020.104103) [104103](https://doi.org/10.1016/j.envexpbot.2020.104103).

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