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Competitive interactions affect larval survival of two root-boring weed biological control candidates of *Rumex* spp

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Abstract In weed biocontrol, the release of multiple candidates has been proposed as a way to increase herbivore load and thus impact on the target weed. However, the use of multiple herbivorous species may reduce overall herbivore load due to interspecific interactions. We assessed whether a combined application of two *Pyropteron* spp. (Lepidoptera: Sesiidae) with root-boring larvae can increase larval infestation rate and impact on two *Rumex* weeds (Caryophyllales: Polygonaceae). In a laboratory assay ('behavioural experiment'), interference competition was assessed by comparing the survival of a single larva (no competition), of four conspecific larvae (intraspecific competition) and of two larvae of each species (interspecific competition). In a common garden study ('infestation and impact experiment'), the outcomes of competitive interactions were assessed on potted plants with single and mixed species applications of the two *Pyropteron* species using a replacement design. In the behavioural experiment, larval survival was similarly reduced under intra- and interspecific competition compared to no competition, suggesting strong interference competition among larvae independent of the *Pyropteron* species. In the infestation and impact experiment, mixed application did only scarcely increase herbivore load. Increased herbivore load was found to increase root decay when applied in an inundative approach by placing eggs directly on *Rumex* plants. However, a joint application of two *Pyropteron* species did not increase the impact. We propose that detailed behavioural and impact studies be conducted to assess the likely outcome of using multiple candidates in biocontrol programs to minimize associated risks with multiple species introductions.

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Introduction

The overall goal of successful weed biocontrol is to build up high herbivore densities that suppress the competitiveness, growth or reproduction of the target weed (Gassmann 1996; Kaplan and Denno 2007; McFadyen 1998; Schwarzländer et al. 2018). To obtain high abundances of biocontrol candidates and therefore a high top-down pressure, the use of multiple candidates has been widely discussed (Denoth et al. 2002; Myers 1985; Myers et al. 1989; Stephens et al. 2013). Linking the biodiversity-ecosystem functioning relationship to biocontrol (Crowder and Jabbour 2014), the potential benefits of introducing multiple candidates against the same target weed can arise through complementarity and/or sampling effects (Loreau and Hector 2001). Functional complementarity refers to niche differentiation or facilitation between species at different scales, with an increase in overall functional performance of the combined species relative to the functional performance of the individual species. The sampling effect refers to selection processes leading to the dominance of a species with the most adapted traits, with an overall performance of the combination of species being equal to the performance of the most adapted species (Loreau and Hector 2001). These biodiversity effects relate to topics discussed in the biocontrol community since the mid-1980s, with the complementarity effect corresponding to the cumulative stress hypothesis (Harris 1985) and the selection/sampling effect to the lottery principle (Myers 1985; Myers et al. 1989).

However, releasing multiple biocontrol candidates may increase the risks of direct or indirect non-target effects (Pearson and Callaway 2003). To keep the potential for undesirable non-target effects as low as possible, it was proposed to introduce the potentially most effective species out of multiple candidates with the help of adapted protocols (Louda et al. 2003). Furthermore, the use of multiple biocontrol candidates targeting the same weed can lead to competitive interactions among the candidates, which can be deleterious for the overall impact on the weed and thus for the likelihood of successful biocontrol, e.g. if the superior competitor is not necessarily the species that can damage the target weed the most (McEvoy and Coombs 1999), or if interspecific competitive interactions negatively affect overall herbivore load (Ehler and Hall 1982). Interspecific interactions between

herbivores are frequent, with competitive interactions being far more common than facilitation interactions (Denno et al. 1995; Kaplan and Denno 2007). Interspecific competitive interactions between herbivores can be driven by indirect competitive interactions, such as plant- or natural enemy-mediated alterations, or by direct competitive interactions in the form of exploitative competition or interference competition. According to Kaplan and Denno (2007), indirect interactions are likely to be more frequent among herbivorous insects than direct interactions.

In weed biocontrol, the effectiveness of the biocontrol candidates is assumed to be, among others, a function of their abundance (McClay and Balciunas 2005; Zalucki and van Klinken 2006). Yet, there is a lack of studies assessing the compatibility of biocontrol candidates pre-release, which may hamper the overall impact and success of such programmes (Buckley et al. 2005; Morin et al. 2009; Schooler and McEvoy 2006; Schwarzländer et al. 2018).

The perennial plant *Rumex obtusifolius* L. (broadleaved dock) is a problematic weed both in its native range in Europe as well as in its introduced range, e.g. in Africa, North and South America, Eastern Australia and New Zealand (Allen 1974, 1975; Cavers and Harper 1964; Zaller 2004), while the South European/North African species *Rumex pulcher* L. (fiddle dock) is invasive in southwestern Australia (Allen 1974, 1975). Two *Pyropteron* species, *Pyropteron doryliforme* (Ochsenheimer) and *Pyropteron chrysidiforme* (Esper) (Lepidoptera; Sesiidae), were considered as classical biocontrol candidates of introduced *Rumex* species in Australia (Scott and Sagliocco 1991a, 1991b). The larvae of these congeneric biocontrol candidates are root borers and their host range is restricted to the genus *Rumex* (Scott and Sagliocco 1991a, 1991b). Ultimately, only *P. doryliforme* was introduced in Australia, because it was able to synchronize its univoltine life cycle to the southern hemisphere conditions, and this species managed to significantly reduce *R. pulcher* densities in Australia (Fisher et al. 1994).

More recently, the same two *Pyropteron* species have been proposed for augmentative biocontrol of *Rumex obtusifolius* in its native range in Europe (Grossrieder and Keary 2004). However, observational and post-release studies in the introduced and in the native range revealed that, despite high numbers of eggs applied, only a low number

of *Pyropteron* larvae were recovered per *Rumex* plant (Hahn et al. 2016; Scott and Sagliocco 1991a, 1991b; Spafford et al. 2008). Possible reasons for the limited attack rate under field conditions may be predation or parasitism of eggs or of freshly hatched larvae. Parasitism of larvae has been reported for both *Pyropteron* species in their native range (Scott and Sagliocco 1991a, 1991b), and for *P. doryliforme* in the introduced range (Spafford et al. 2008). Yet, these factors alone are unlikely to explain the low number of early-instar larvae retrieved per plant. Both Spafford et al. (2008) and Hahn et al. (2016) suggested that the low number of larvae retrieved per plant may be due to intraspecific competition.

As in numerous other weed biocontrol programs, so far the two *Pyropteron* species and their impact on *Rumex* plants have been studied in isolation. However, the two species are prone to interspecific interference competition, as this type of competition is particularly common under spatiotemporal co-occurrence between closely related species, between mandibulate herbivores and when the species share concealed niches (Denno et al. 1995). Therefore, there is a need to assess the potential of interspecific competition between larvae of these two biocontrol candidates and to compare it with the level of competition among individuals of the same species (intraspecific competition). Furthermore, the degree of competition should be assessed on different target weeds, as plant traits can mediate competition among herbivorous insects (Kaplan and Denno 2007).

The objectives of this study were to assess the potential of increasing herbivore load and impact on *Rumex* spp. by applying the two sesiid biocontrol candidates jointly, rather than singly, and to elucidate whether an increase or lack of increase in herbivore load and impact can at least partly be explained by intra- and/or interspecific competition. To this aim, we conducted a behavioural experiment and an infestation and impact experiment to assess whether (1) the survival of jointly applied larvae is lower than that of larvae applied individually, (2) the survival of jointly applied larvae depends on whether the larvae are from the same (intraspecific) or from different species (interspecific), (3) the impact on *Rumex* plants is affected by increasing numbers of larvae per plant, and whether (4) the two biocontrol candidates differ in their infestation and impact on *Rumex* plants.

Materials and methods

Study species

The two clearwing moths *P. chrysidiforme* and *P. doryliforme* are univoltine species with root-boring larvae. Both species are native to Europe but differ in their range of climatic preference: while *P. doryliforme* is native to the western Mediterranean region of southern Europe and North Africa, *P. chrysidiforme* has a central and southern European distribution (Scott and Sagliocco 1991a, 1991b).

Pyropteron chrysidiforme individuals were obtained from a rearing colony, which was set up in 2010 with insects collected in south-western Switzerland (rearing at CABI Switzerland Centre, Delémont). *Rumex* plants with late-instar larvae of *P. doryliforme* were collected in April 2018 in southern Spain and transferred to CABI Switzerland to also establish a rearing colony. Between 30 May and 1 July 2018 and between 30 June and 17 July 2020, freshly laid eggs were transported weekly from the CABI Centre to Agroscope, the Swiss centre of agricultural research, in Zürich-Reckenholz to set up the behavioural and the infestation and impact experiments.

Rumex pulcher and *R. obtusifolius* are perennial hemicryptophyte plant species in the family Polygonaceae. Both species are characterized by having a fleshy taproot, a basal rosette and a high seed production. *Rumex obtusifolius* seeds were collected in 2016 in the vicinity of the CABI Center in Delémont, and *R. pulcher* seeds were provided by the botanical garden of the University of Konstanz, Germany, in 2011. All seeds were stored in a refrigerator at 8° C until sowing.

Experimental design and measurements

Behavioural experiment

This experiment aimed to assess the survival of larvae transferred individually or in groups on sections of *Rumex* roots. In late June-early July 2018, a four-factorial behavioural experiment was set up in Petri dishes in the laboratory facilities of Agroscope at Zürich-Reckenholz. Larval survival, defined here as the number of larvae retrieved alive after a certain amount of time compared to the number of larvae initially transferred, was assessed for both

Pyropteron species, *P. doryliforme* and *P. chrysidiforme* (first factor), at three competition treatments (second factor): (1) no competition (one larva per Petri dish), (2) intraspecific competition (four conspecific larvae per Petri dish), and (3) interspecific competition (two larvae each of the two species per Petri dish). Thus, intra- and interspecific competition treatments followed a replacement design. These treatment combinations were applied on two *Rumex* species (third factor) and were set up three times (fourth factor) to allow for evaluation of the temporal development.

For the no competition treatment, a single larva of one *Pyropteron* species was transferred within 24 h after hatching onto one root section of approximately 1.5 cm length and diameter of 0.5 cm of either *R. obtusifolius* or *R. pulcher* wrapped in moist paper tissue, and the root section was placed in a Petri dish. The same procedure was applied for the intraspecific and interspecific competition treatments, but the four larvae were transferred within a few minutes on a root section of approximately 4 cm length and diameter of 0.5 to 0.6 cm width to permit successful encounter of the larvae. The larger root sections used in the competition treatments guaranteed that the larvae were not suffering from food shortage during the experiment. The Petri dishes were closed with medical tape and randomly placed on a laboratory desk at ambient room conditions. Paper moisture was checked twice a week and, if necessary, re-moisturised using a vaporiser. In July 2020, the whole setup was repeated to increase the number of replicates. The no competition treatment (single application) was finally replicated at least 25 times for each treatment combination (*Rumex* species × *Pyropteron* treatment × treatment time) and the intra- and interspecific competition treatments at least 15 times. Different numbers of replicates were set up to balance to some degree the number of larvae evaluated in the different competition treatments (one larva per sample in the no competition treatment *versus* four larvae per sample in the competition treatments). After five, ten and 15 days, respectively, one third of the Petri dishes were checked for the number of larvae alive, dead or missing. The restriction to 15 days was taken because the first instar stages are most prone to competition. This time is maximally required to attain the second instar and start tunnelling into the root (see Scott and Sagliocco 1991b).

Infestation and impact experiment

In 2018, the effect of single and combined application of *P. chrysidiforme* and *P. doryliforme* on larval infestation and impact was assessed in a three-factorial pot experiment established in a common garden at Zürich-Reckenholz (47°43'N, 8°52' E). Three *Rumex* treatments (first factor): (1) *R. obtusifolius* seed-grown, (2) *R. pulcher* seed-grown, and (3) *R. obtusifolius* taproot were subjected to four *Pyropteron* treatments (second factor): (1) control (no infestation; Ctr), (2) *P. chrysidiforme* single species application (Pch), (3) *P. doryliforme* single species application (Pdo), and (4) *P. chrysidiforme* and *P. doryliforme* mixed application (Pch-Pdo mixed). *Pyropteron* application followed a replacement design. To assess infestation rate and impact before the winter and in the following spring, plants were either harvested in autumn 2018 or in spring 2019 (third factor). *Rumex* × *Pyropteron* treatment combinations were set up with 15 replicates per harvest season, resulting in 360 plants.

In April 2018, seedlings of *R. obtusifolius* and *R. pulcher* were reared and planted in pots (volume 5.5 l, diameter 19.5 cm, height 25.5 cm) containing a mixture of horticultural soil, sand, vermiculite and commercial slow-release fertilizer. To include plants with naturally developed root systems (taproot with secondary roots), plants of *R. obtusifolius* were collected in a meadow near Porrentruy, Switzerland, where neither of the two *Pyropteron* species naturally occur. Roots were washed and transplanted into pots as described above. Young plants were first cultured in a glasshouse at ambient temperature and photoperiod, and watered as required (temperature mean 21.5 °C and range 12.9–37.0 °C; natural and supplementary light provided between 7:00 and 22:00). In early June, potted plants were translocated to the common garden of Agroscope at Zürich-Reckenholz. Plants were arranged in a randomized complete block design, with 15 blocks of 24 plants set up in six rows and four columns. The plants were exposed to natural rainfall but were watered during an extreme drought event in mid-July 2018.

In mid-June 2018, *Rumex* plants were inoculated with the biocontrol candidates by inserting a toothpick with 30 eggs glued on into the soil next to the plant base, as described in Hahn et al. (2016). Prior to inoculation, above-ground biomass was cut at 6–7 cm

above ground to allow similar conditions concerning sun irradiation and to mimic management. For the *Pyropteron* single species application, plants were inoculated with 30 eggs of the corresponding *Pyropteron* sp. In the mixed application treatment, plants were inoculated with 15 eggs of each of the two *Pyropteron* species. Inoculation of plants occurred between 13 and 27 June 2018. While inoculation of plants from the same block occurred on the same day, the timing of the inoculation of blocks depended on the availability of freshly laid eggs. Plants and egg sticks were protected from rainfall during two weeks after inoculation.

Plants were harvested, the fresh aboveground biomass removed, and roots dissected block-wise between 17 September and 11 November 2018 and the following spring between 1 and 24 April 2019. After carefully removing the soil from the roots, these were weighed and the number of roots recorded. Roots were subsequently dissected to determine the number of *Pyropteron* larvae (total, alive and dead). To assess the infestation probability, we considered a plant as infested if at least one alive or dead larva was recovered. A measure of herbivore load in relation to *Rumex* plant performance was calculated by dividing the total number of larvae by final root mass. To further assess the impact of the biocontrol candidates on the *Rumex* plants, the proportion of the root decayed (dead material, structure porous, color of root material blackish, brownish or redish, differing from healthy root color) was estimated visually and attributed to one of six percentage categories (0, 5, 15, 50, 85, 100%).

Data analysis

In the behavioural experiment, two generalised linear models (GLMs) were used to analyse the survival probability of larvae. First, we analysed survival of larvae (1 if alive, 0 if dead) as affected by intraspecific *versus* no competition (factor with two levels) for each of the two *Pyropteron* species (factor with two levels), applied on the two *Rumex* species (factor with two levels) and assessed three times (factor with three levels) (subset of data without the interspecific competition treatment). The model used the logit link function and included all main effects and two-way interactions. Inference on main effects was determined by single term deletion from the main effects

model (each effect in turn) and subsequent likelihood ratio tests. Interactions were similarly tested, but from a model that included all two-way interactions. Driven by the research questions, specific differences in the survival probability between the factor levels of variables were tested based on the main effects models' contrasts (post-hoc Wald z-tests, without applying multiple comparisons) because all interactions (also those of higher order) turned out to be not significant.

In a second GLM, we evaluated survival of larvae as affected by intraspecific *versus* interspecific competition (subset of data without the no competition treatment). Explanatory factors were competition (factor with two levels: intraspecific *versus* interspecific competition), *Rumex* species and time (as defined). Note that this second model was needed to appropriately analyse the interspecific competition treatment because it was not orthogonally related to the two *Pyropteron* species. The model specification and testing of variables and their factor levels was done as explained above, and a detailed description of the model equations is given in Supplementary Appendix S1. The R^2-values of both GLMs were calculated following Nagelkerke ([1991](#)). Moreover, the effect of the examination year (2018, 2020) was initially tested in both GLMs. Yet, survival of larvae was similar across *Rumex* species and *Pyropteron* treatments in both years, and so this was not further considered.

Data gained from the infestation and impact experiment were analysed with (generalised) linear mixed-effects models. Each of seven response variables, namely infestation, total number of *Pyropteron* larvae retrieved, total number of larvae scaled by root mass, number of larvae retrieved alive, root mass of *Rumex* roots, proportion of root decay, and number of *Rumex* roots were modelled as a function of the same predictors, namely *Rumex* treatment (fixed factor with three levels), *Pyropteron* treatment (fixed factor with four levels), harvest season (fixed factor with two levels), and the block effect (random factor modelled as intercept). Yet, the different underlying distributions of response variables required the use of a variety of link functions (see Supplementary Appendix S1 and Table S1 for details). The final model included all main effects and two-way interactions. The inference on model terms was determined by single term deletion as described, and tests between factor levels of

Table 1 Summary of generalised linear models for the effects of **a** *Rumex* species, *Pyropteron* species, no *versus* intraspecific competition, and treatment time, and of **b** *Rumex* species, intraspecific *versus* interspecific competition, and treatment time on the survival probability of *Pyropteron* larvae in the behavioural experiment. See Supplementary Tables S2 and S3 for the model summary including all two-way interactions (all being not significant)

Variable	df	χ^2	p
(a) No *versus* intraspecific competition			
Rumex species	1	1.2	0.282
Pyropteron species	1	5.5	0.019
Competition (no *vs.* intraspecific)	1	115.5	<0.001
Time	2	49.1	<0.001
R^2		0.291	
(b) Intra *versus* interspecific competition			
Rumex species	1	1.0	0.307
Competition (intra- *vs.* interspecific)	1	1.2	0.269
Time	2	36.9	<0.001
R^2		0.120	

R^2: Maximum rescaled R^2 following Nagelkerke (1991)

variables were done by post-hoc Wald tests from the two-way interactions model (without applying multiple comparisons). The marginal and conditional R^2 of models were calculated following Nakagawa and Schielzeth (2013) and Nakagawa et al. (2017). All data were analysed with the statistics software R, version 4.1.2 (R Core Team 2021) and the glmmTMB package for generalised linear mixed-effects models (Brooks et al. 2017).

Results

Survival of *Pyropteron* larvae in the presence and absence of competition

In the behavioural experiment, average larval survival was significantly higher (Table 1a) in Petri dishes containing a single larva (larval survival of 0.76 ± 0.02) than in Petri dishes containing four conspecific *Pyropteron* larvae (larval survival of 0.44 ± 0.03; Fig. 1). Larval survival was significantly
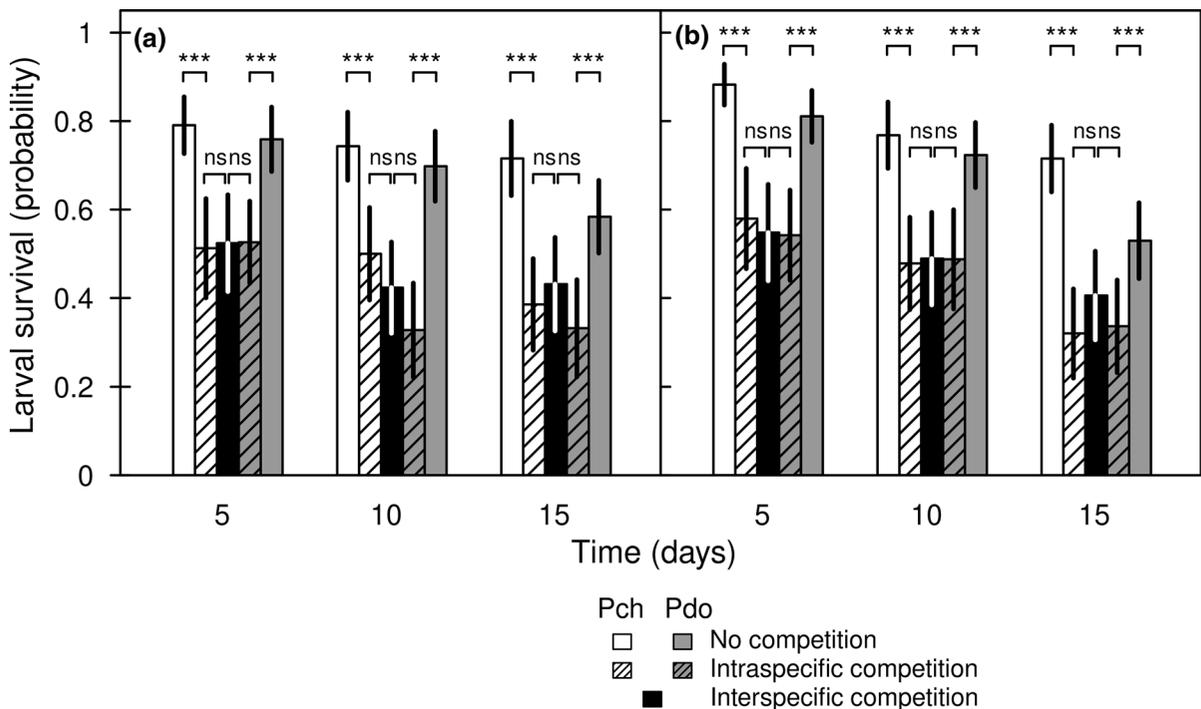

Fig. 1 Survival of *P. chrysidiforme* (Pch) and *P. doryliforme* (Pdo) larvae feeding on roots of *R. obtusifolius* (**a**) and *R. pulcher* (**b**) as affected by *Pyropteron* application treatments (no competition, intraspecific and interspecific competition) and time in the behavioural experiment. Displayed are means $\pm$ SE, calculated following Agresti and Coull (1998). Tests are based on generalised linear models (Tables 1a and 1b). ***p $\leq$ 0.001, ns: not significant

higher for *P. chrysidiforme* (without competition: 0.80 ± 0.03; with competition: 0.45 ± 0.05) than for *P. doryliforme* (without competition: 0.71 ± 0.03; with competition: 0.43 ± 0.05) but did not differ between the two *Rumex* species (Table 1a). Furthermore, larval survival was significantly affected by and decreasing over assessment time, with average survival probabilities across *Rumex* species, *Pyropteron* species and competition treatment of 0.71 ± 0.03, 0.61 ± 0.03 and 0.51 ± 0.03 after 5, 10 and 15 days, respectively (Table 1a, Fig. 1).

Single *versus* multiple species competitive interactions

In the behavioural experiment, no significant differences were observed in larval survival where either four conspecific larvae or two larvae of each of the two *Pyropteron* species were transferred on a root piece, irrespective of the *Pyropteron* species (Table 1b, Fig. 1). On average, larval survival was 0.44 ± 0.03 and 0.46 ± 0.05 for the intraspecific and interspecific competition treatments, respectively. No treatment interactions were observed in the behavioural experiment (Supplementary Tables S2 & S3).

Similarly, in the infestation and impact experiment, generally no differences were found between single species application (irrespective of *P. chrysidiforme* or *P. doryliforme*) and mixed application regarding infestation probability (Fig. 2a–c) and total number of larvae retrieved per infested plants (Fig. 2d–f). In those cases where distinct differences between single and mixed applications of *Pyropteron* species were found, the mixed treatment performed better than single application of *P. chrysidiforme* and/or *P. doryliforme* (Fig. 2b and d). Infestation probability of plants subjected to *Pyropteron* application was significantly affected by harvest time (Table 2, Fig. 2a–c), with the infestation probability being higher in autumn 2018 (0.67 ± 0.07) than in spring 2019 (0.54 ± 0.07; across all *Pyropteron* and *Rumex* treatments).

In the infestation and impact experiment, a lower number of larvae per infested plant was found on *R. pulcher* than on *R. obtusifolius* (*P. chrysidiforme*: 1.8 ± 0.38, 3.9 ± 0.44 and 4.2 ± 0.70 larvae on *R. pulcher* seed-grown, *R. obtusifolius* seed-grown, *R. obtusifolius* taproot, respectively; *P. doryliforme*: 1.7 ± 0.33, 2.9 ± 0.40 and 3.2 ± 0.48 larvae on the three *Rumex* treatments, respectively; Table 2, Fig. 2d-f). However, when the total number of larvae retrieved was scaled by the root mass of the *Rumex* plants, more larvae per unit biomass were retrieved from *R. pulcher* seed-grown than from *R. obtusifolius* seed-grown (Supplementary Figure S1 and Supplementary Table S4). The proportion of larvae alive was on average 0.91 ± 0.05, indicating that the great majority of larvae was alive at the time of dissection. The proportion of larvae alive ranged from 0.82 to 1.00 and depended on the *Rumex* and *Pyropteron* treatments (compare Fig. 2d–f and Supplementary Figure S2, no extra figure on proportions of larvae alive).

Impact of *Pyropteron* larvae

Although infestation probability and number of larvae per plant were relatively high in the infestation and impact experiment, root mass was only impacted in autumn 2018 for seed-grown plants. Relative to the control, root mass of *Pyropteron*-treated plants was 36.5% lower for *R. obtusifolius* seed-grown (Fig. 3a) and 34.2% lower for *R. pulcher* seed-grown plants (Fig. 3b, Table 2). Moreover, in autumn 2018, *R. pulcher* plants subjected to *Pyropteron* treatments had a lower number of roots than plants from the control group, while *R. obtusifolius* seed-grown and taproots under *Pyropteron* treatments had more roots in spring 2019 (Supplementary Figure S3). Similarly, *R. obtusifolius* taproots treated with *Pyropteron* had higher mass in spring 2019 than control plants (Fig. 3c).

All *Rumex* plants showed a significantly higher degree of proportional root decay under *Pyropteron* application than in the control treatment and this effect was significant over both seasons (Table 2, Fig. 3d–f). Across inoculated plants and harvest seasons, proportional root decay was 0.22 ± 0.03, 0.29 ± 0.05 and 0.50 ± 0.05 for *R. obtusifolius* seed-grown, *R. pulcher* seed-grown and *R. obtusifolius* taproot, respectively, compared to 0.07 ± 0.01, 0.06 ± 0.01, and 0.26 ± 0.06 in the control treatment (Table 2, Fig. 3d–f). For root mass and proportion of root decay, generally no differences were observed between single species application of either *P. chrysidiforme* or *P. doryliforme* and mixed application (Fig. 3a–c & Fig. 3d–f).

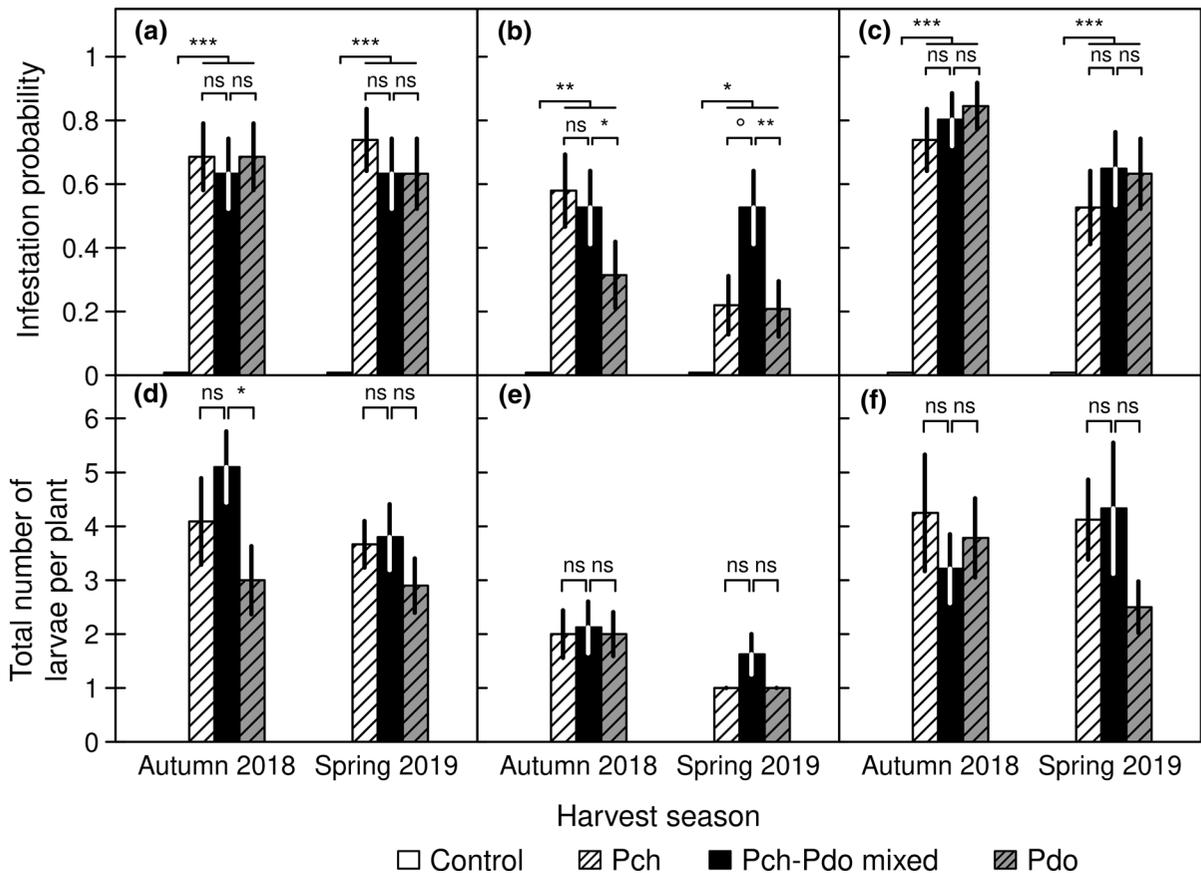In all *Rumex* treatments, the proportion of root decay was positively related to the number of

Fig. 2 Probability of infestation by least one larva (**a**, **b**, **c**) and total number of larvae retrieved from plants (**d**, **e**, **f**) of different *Rumex* treatments (*R. obtusifolius* (**a**, **d**), *R. pulcher* (**b**, **e**), *R. obtusifolius* taproots (**c**, **f**)) as affected by *Pyropteron* application treatments (no infestation [Control] (only for infestation probability), *P. chrysidiforme* application [Pch], *P. doryliforme* application [Pdo], mixed application [Pch-Pdo mixed]) and harvest season in the infestation and impact experiment. Displayed are means ± SE, calculated following Agresti and Coull (1998) in a-c. Only infested plants displayed in d-f, and tests are based on generalised linear mixed-effects models (Table 2). ***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05, p = 0.083 (i.e., ns), ns: not significant

larvae retrieved (Fig. 4). Yet, the degree of this relationship differed between *Rumex* treatments (Fig. 4: *Rumex* × slope interaction: $\chi^2 = 16.25$, df = 2, p < 0.001), but was not affected by the different *Pyropteron* applications (z ≤ 0.61, p > 0.545). However, when the total number of larvae retrieved was scaled by the final root mass, the relationship to the proportion of root decay became similar for all three *Rumex* treatments (Supplementary Figure S4: *Rumex* × slope interaction: $\chi^2 = 1.50$, df = 2, p = 0.472). Again, there was no effect of the

different *Pyropteron* applications on this relationship (z ≤ 0.24, p > 0.813).

Discussion

Our results provide evidence that a joint application of the two biocontrol candidates *P. chrysidiforme* and *P. doryliforme* does not increase herbivore load or impact on *Rumex* plants due to competitive interactions among the larvae. The behavioural experiment revealed reduction of larval survival under

Table 2 Summary of generalised linear mixed-effects models for the effects of *Rumex* treatment (*Rumex*), *Pyropteron* treatment (Pyropt_treat), and harvest season on response variables related to the fitness of applied *Pyropteron* species in the infestation and impact experiment. For 'Total number of larvae' only infested plants were included in the analysis. The three-way interaction was never significant (p > 0.3, each variable). See Supplementary Appendix S1 and Table S1 for the link functions of all response variables

Variable	Infestation probability			Total number of larvae[§]			Root mass			Proportion root decay		
	df	χ^2	p	df	χ^2	p	df	χ^2	p	df	χ^2	p
Rumex treatment	2	36.9	< 0.001	2	44.5	< 0.001	2	225.2	< 0.001	2	95.0	< 0.001
Pyropteron treatment	3	115.4	< 0.001	2	5.8	0.054	3	5.0	0.170	3	98.8	< 0.001
Season	1	6.7	0.009	1	2.5	0.111	1	0.5	0.499	1	< 0.1	0.914
Rumex × Pyropt_treat	6	13.5	0.036	4	2.1	0.711	6	11.1	0.086	6	6.8	0.340
Rumex × Season	2	4.0	0.132	2	2.0	0.372	2	0.8	0.683	2	0.6	0.748
Pyropt_treat × Season	3	2.7	0.436	2	1.1	0.578	3	11.3	0.010	3	3.0	0.399
R^2_m		0.465			0.279			0.630			0.292[‡]	
R^2_c		0.608			0.445			0.686			0.310[‡]	

[§]No larvae were retrieved in the control treatment. Controls were thus omitted from the analysis and df related to the *Pyropteron* treatment accordingly reduced

[‡]Approximation by squared correlation of linear predictor and link-transformed response

R^2_m and R^2_c: marginal and conditional R^2, respectively, following Nakagawa and Schielzeth (2013) and Nakagawa et al. (2017). R^2_m: variance explained by fixed effects; R^2_c: variance explained by fixed and random effects

both intraspecific and interspecific competition, compared to no competition. In the infestation and impact experiment, joint application of the two biocontrol candidates did not increase consistently the infestation rate compared to single species application with either of the two *Pyropteron* species, and the two treatments only marginally differed in the number of larvae retrieved. Furthermore, the attack levels remained generally low with respect to number of eggs applied and root (resource) availability, suggesting that competitive interactions observed in the behavioural experiment also occurred in this semi-natural setting. Thus, while root decay of *Rumex* plants was positively related to the number of larvae retrieved, a combined release of the two biocontrol candidates is unlikely to increase herbivore load and thus impact on either *R. obtusifolius* or *R. pulcher*.

Intra- *versus* interspecific interference competition between *Pyropteron* species

Results from both experiments suggest that the strong intra- and interspecific competition among larvae of the two biocontrol candidates *P. chrysidiforme* and *P. doryliforme* was driven by interference competition, i.e. by direct, physical interactions between larvae. Larvae were repeatedly observed to display aggressive behaviour upon encounter during the behavioural experiment (Klötzli, pers. observation). Several of these encounters led to the death of one of the larvae, which was subsequently sometimes eaten by the surviving larva. Other forms of competition are unlikely to significantly contribute to the results obtained in our experiments. First, we found no relationship between root mass and number of larvae retrieved per plant, suggesting that at least the number of larvae retrieved from larger plants was not affected by exploitative competition, i.e., defined here as a competition that occurs when one species consumes and thus reduces the quantity of resources available to competitors (Kaplan and Denno 2007). Second, although parasitism of *Pyropteron* larvae was observed under natural conditions (Scott and Sagliocco 1991a, 1991b; Spafford et al. 2008), enemy-mediated interactions are unlikely to be the principal mechanism explaining the low number of larvae retrieved per plant in our study, as no predation or parasitism was detected in either of the two experiments.

Although interference competition does not seem to be widespread among herbivores (Kaplan and Denno 2007), some characteristics of herbivore species appear to increase the likelihood of direct aggressive interactions. Denno et al. (1995)
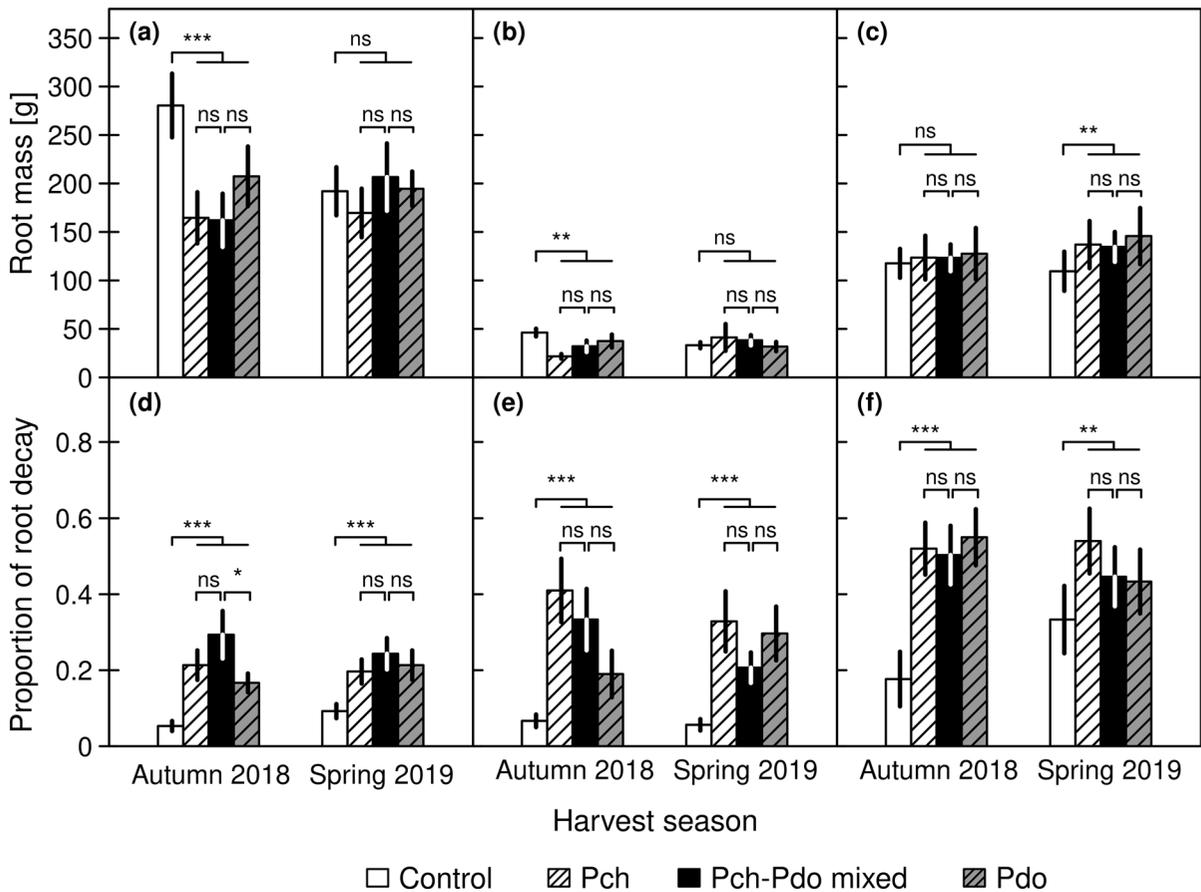
Fig. 3 Root mass (**a**, **b**, **c**) and proportion of root decay (**d**, **e**, **f**) of different *Rumex* treatments (*R. obtusifolius* (**a**, **d**), *R. pulcher* (**b**, **e**), *R. obtusifolius* taproots (**c**, **f**)) as affected by *Pyropteron* application treatments (no infestation [Control], *P. chrysidiforme* application [Pch], *P. doryliforme* application [Pdo], mixed application [Pch-Pdo mixed]), and harvest season in the infestation and impact experiment. Displayed are means ± SE. Tests are based on generalised linear mixed-effects models (Table 2). ***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05, ns: not significant

suggested that interspecific interference competition is more common among herbivores when they live in the same concealed niche, are mandibulate herbivores, and when they are closely related. The two biocontrol candidates *P. chrysidiforme* and *P. doryliforme* share all these characteristics. It is thus not surprising that their interaction was driven by interference competition, and our results support earlier notions that larval densities of *P. chrysidiforme* and *P. doryliforme* may be regulated by intraspecific competition (Hahn et al. 2016; Spafford et al. 2008). Still, under field conditions, on a few occasions considerably higher infestation rates were observed, with up to 10 and 32 larvae per infested root for *P.*

chrysidiforme and *P. doryliforme* (Hahn et al. 2016; Spafford et al. 2008), respectively. It remains to be explored under which circumstances such high attacked rates may occur. One possible explanation is that eggs were laid on these plants over an extended period, which may reduce encounters of early instars and buffer against unsuitable weather conditions when freshly hatched larvae crawl down the plant and try to enter the root. We acknowledge that the female oviposition behaviour largely determines the spatiotemporal distribution of eggs and larvae in naturally occurring butterfly populations and thus the likelihood of intra- or interspecific larval encounters. In our study, however, we focused on a management approach in which the biocontrol
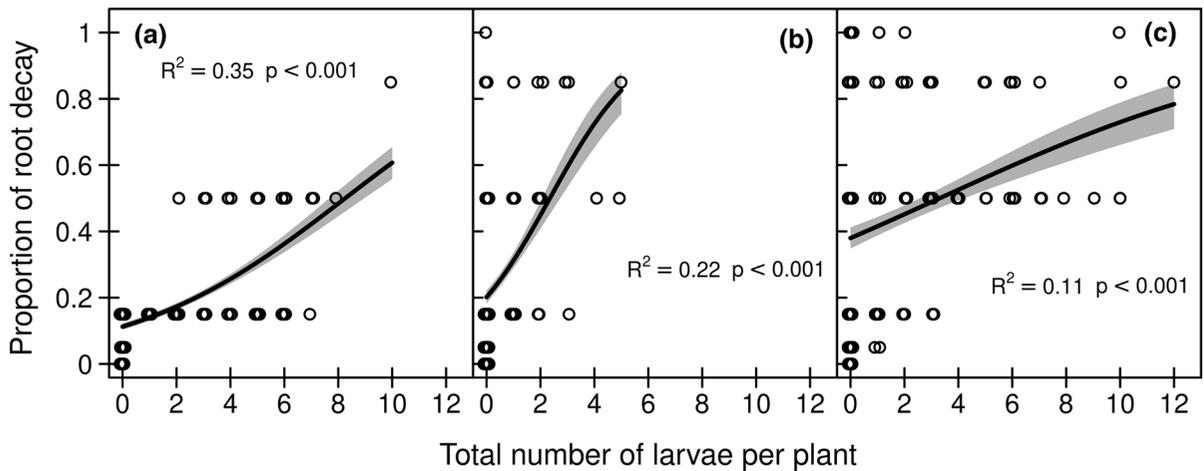
Fig. 4 Proportion of root decay of different *Rumex* treatments (*R. obtusifolius* (**a**), *R. pulcher* (**b**), *R. obtusifolius* taproots (**c**)) depending on the total number of larvae per infested plant in the infestation and impact experiment. Predicted lines (± SE grey shaded), R² and p-values are based on beta regression. Circles are scattered horizontally to improve their visibility

agents are applied at the egg stage, as implemented in Australia (Spafford et al. 2008) and tested in Europe (Hahn et al. 2016).

Implications for biocontrol of *Rumex* using *Pyropteron* species

The relative reduction in root mass of seed-grown *R. obtusifolius* and *R. pulcher* three months after inoculation with *Pyropteron* eggs were similar to below-ground biomass reductions reported by Blossey and Hunt-Joshi (2003) for other root-feeding biocontrol candidates. However, in spring 2019 the effect of herbivory on root mass was not significant anymore and *R. obtusifolius* taproots had even (slightly) more mass. This may be explained by the fact that the plants in the infestation and impact experiment grew without plant competition and may thus have been able to compensate for herbivore damage during autumn and winter. A greater and long-lasting reduction in root biomass by herbivory is expected when top-down pressure is combined with interspecific plant competition as an additional stress factor (Hambäck and Beckerman 2003). In contrast to root biomass, we found in both seasons a significantly increased root decay in all *Pyropteron* treatments, relative to the control treatment. The discrepancy between the inconsistent impact on root mass and high proportion of root decay was likely due to decayed root material that was also included in the assessment of root biomass, which may lead to erroneous conclusions regarding the impact of *Pyropteron* on 'functioning' root biomass.

The slope of the positive relationships between herbivore load and proportion of root decay was flatter for the *R. obtusifolius* than for the *R. pulcher* plants (Fig. 4). However, when herbivore load was scaled by the final root mass, no difference was found among *Rumex* treatments (Supplementary Figure S4). One may therefore speculate that populations of *R. pulcher* were successfully controlled by *P. doryliforme* in Australia because *R. pulcher* roots tend to be smaller (Spafford et al. 2008) and therefore fewer larvae were needed to achieve significant root decay. The high proportion of root decay also facilitates secondary infection by fungi, which may further enhance impact under natural conditions (Caesar 2003).

Our study suggests that using two *Pyropteron* species in an inundative approach does not lead to a generally higher herbivore load than single species applications, as one would expect in the case of a complementary effect. We detected only one case where the mixed treatment performed better than either of the single species applications (Fig. 2b), while in the other cases, the mixed treatment performed better than *P. doryliforme*. These results suggest a sampling effect happening through selective processes causing high relative

abundance of a species with specific traits (Loreau and Hector 2001), with the overall functional performance largely attributable to *P. chrysidiforme*. Although in our experiments the application of the two *Pyropteron* species did not improve herbivore load or impact on the target weed, the use of multiple candidates in an inundative biocontrol approach against *Rumex* spp. should not generally be discarded. For example, the efficacy of biocontrol may be increased using biocontrol candidates with different spatial niches (Stephens et al. 2013). Grossrieder and Keary (2004) proposed a list of potential candidates against *R. obtusifolius,* some of which occupy different feeding niches than the *Pyropteron* species.

Testing for (positive or negative) interactions among biocontrol candidates

Biocontrol candidates which are released against the same target weed may interact, which could significantly affect the outcome of the biocontrol programme. While synergistic or additive outcomes would be desirable, interspecific competition among herbivores can cause antagonistic or inhibitive effects (Denoth et al. 2002; Fournier et al. 2006; but see Hatcher 1996). Although in numerous biocontrol projects more than one candidate has been released, only a few studies have looked at interaction effects pre-release. For example, pre-release experiments on impact of pairwise combinations of different biocontrol candidates were made on water hyacinth (Marlin et al. 2013), as well as on garlic mustard (Gerber et al. 2008). When single and pair-wise combination of multiple candidates' damage were compared, effects ranged from equivalent (Gerber et al. 2008; Marlin et al. 2013) to synergistic (Marlin et al. 2013), as defined in Fournier et al. (2006). Furthermore, a study by Gerber et al. (2008) has demonstrated similar competitive interactions among multiple biocontrol candidates and equivalent outcomes on plant performance.

Carefully designed pre-release studies, which specifically compare the potential risk of intra- and interspecific competition among biocontrol candidates, will reduce the risk associated with multiple releases of biocontrol candidates against the same target weed. We therefore recommend that an assessment

of positive or negative interactions among biocontrol candidates and the resulting impact on the herbivore load and impact on the weed should be done more routinely in weed biocontrol programs.

Declarations

Research involving human animal participate This article does not contain any studies with human participants or animals (vertebrates) performed by any of the authors.

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