

Experimental test of plant defence evolution in four species using long-term rabbit exclosures

Teresa J. Didiano¹†, Nash E. Turley²†*, Georg Everwand³, Hanno Schaefer⁴, Michael J. Crawley⁵ and Marc T. J. Johnson²

¹Department of Geography, University of Toronto Mississauga, 3359 Mississauga Road, Mississauga, Ontario L5L 1C6, Canada; ²Department of Biology, University of Toronto Mississauga, 3359 Mississauga Road, Mississauga, Ontario L5L 1C6, Canada; ³Department of Crop Sciences, Agroecology, University of Göttingen, Grisebachstrasse 6, Göttingen 37077, Germany; ⁴Biodiversität der Pflanzen, Technische Universität München, Maximus-von-Imhof Forum 2, Freising 85354, Germany; and ⁵Department of Biology, Imperial College at Silwood Park, Ascot, Berkshire SL5 7PY, UK

Summary

1. Plant defence traits have evolved over macro- and microevolutionary time-scales in response to herbivores. Although a number of studies have investigated the evolutionary impacts of herbivores over short time-scales, few studies have experimentally examined what defence traits most commonly evolve and whether multiple coexisting species exhibit similar evolutionary responses to herbivores.

2. We addressed these questions using a long-term experiment at Silwood Park, England, United Kingdom, where we excluded rabbits from 38 grassland plots for <1–34 years. To assess the evolutionary impacts of rabbits on plant defence traits, we collected seeds from plots containing the following perennial species: *Anthoxanthum odoratum* (Poaceae), *Festuca rubra* ssp. *rubra* (Poaceae), *Holcus lanatus* (Poaceae) and *Stellaria graminea* (Caryophyllaceae). We then grew these plants in a common garden and measured defensive and morphological traits.

3. We found some evidence for evolutionary change of defence traits in three of the four species following the exclusion of rabbits. We observed the clearest changes in *F. rubra*, which showed a 9% decline in tolerance to herbivory and a 26% decline in leaf number. We also observed weak evidence for a change in all grass species towards a more erect growth form suggesting that grazing selects for plants that grow close to the ground.

4. Although our results are most consistent with evolution due to changes in the frequency of alleles and genotypes, we cannot rule out that epigenetic changes (e.g. methylation) or maternal environmental effects also contributed to or caused the observed long-term phenotypic responses.

5. *Synthesis.* Our study provides an experimental test of the evolutionary effects of an ecologically important herbivore. We found evidence for plant defence evolution following >20 years of rabbit exclusion. However, the evidence was only strong in one species for multiple traits, weak in all three grass species for avoidance and absent in an herb species. This suggests that the evolutionary effects of an ecologically important herbivore on plants will be variable and difficult to predict in nature.

Key-words: avoidance strategy, contemporary evolution, herbivory, plant–herbivore interactions, Poaceae, tolerance

Introduction

Contemporary evolution, defined as evolution within a population over <1–100 years, is a common phenomenon observed in plant, animal and microbial species (Dyer 1968; Thompson

1998; Bone & Farres 2001; Hendry & Kinnison 2001). For example, plant populations frequently adapt to different environmental conditions within years to decades, even over very small spatial scales (Antonovics & Bradshaw 1970; Snaydon & Davies 1972; Franks, Sim & Weis 2007). While these studies typically focus on adaptation to abiotic factors, species interactions can also cause adaptive evolution in plant populations (Linhart & Grant 1996; Garrido, Andraca-Gomez &

*Correspondence author. E-mail: nashuagoats@gmail.com

†These authors contributed equally to this work.

Fornoni 2012; Galetti *et al.* 2013). Plant–herbivore interactions are important drivers of ecological processes (Strong, Lawton & Southwood 1984; Cyr & Pace 1993; Maron & Crone 2006), and there is mounting evidence that herbivores drive contemporary evolution in plant populations (Detling & Painter 1983; Lennartsson, Tuomi & Nilsson 1997; Zangerl & Berenbaum 2005; Stenberg, Witzell & Ericson 2006; Agrawal *et al.* 2012; Fukano & Yahara 2012; Zust *et al.* 2012; Ågren *et al.* 2013). However, other studies show little or no evolutionary effects of herbivores on plant populations (Fahnestock & Detling 2000; Tomas, Carrera & Poverene 2000; Hornoy *et al.* 2012). Given this, three key questions on the evolution of plant defences remain unresolved: How common is herbivore-driven evolution across multiple plant species within a community? What defence traits most frequently evolve as adaptations in response to natural selection by herbivores? And do coexisting species that experience similar levels and types of herbivory exhibit convergent evolutionary responses to selection?

Plants have a large diversity of defensive traits which are expected to evolve in response to selection by herbivores (Fraenkel 1959; Stamp 2003; Agrawal 2007; Carmona, Lajeunesse & Johnson 2011). A plant defence is any trait that increases plant fitness in the presence of herbivores relative to when herbivores are absent (Karban & Baldwin 1997; Strauss & Agrawal 1999). The three most common categories of plant defences are resistance, avoidance and tolerance. Resistance traits are morphological (e.g. thorns) and chemical (e.g. tannins) traits that deter and reduce herbivore damage. Avoidance is any morphological (e.g. prostrate growth) or phenological (e.g. bud break) trait that enables plants to escape damage in either space or time (Strauss & Agrawal 1999; Walters 2011). By contrast, tolerance reduces the negative fitness effects of herbivore damage and includes traits such as compensatory growth and increased photosynthetic rates following herbivory (Strauss & Agrawal 1999; Stowe *et al.* 2000). A number of studies have demonstrated short-term evolution of plant defence traits in response to herbivores. For example, Detling & Painter (1983) observed the evolution of decreased tolerance to herbivory and increased height in western wheatgrass following the experimental removal of prairie dogs. Similarly, Agrawal *et al.* (2012) experimentally manipulated the presence of insect herbivores on evening primrose and observed the evolution of later flowering and decreased chemical defences in populations lacking insects. However, not all studies show the evolution of plant defence traits in response to herbivores. For example, Tomas, Carrera & Poverene (2000) collected seeds from areas with and without mammalian grazing and found no difference in morphological traits when seeds were grown in a common environment. These studies provide evidence that herbivores can impose selection on plant defence traits leading to contemporary evolution in plant populations, but this is not the case for all plant–herbivore interactions.

Mammalian grazing herbivores are predicted to be among the most important agents of natural selection on plants (McNaughton 1984; Pollard 1992). Grazers typically have

broad diets and larger effects on individual plant fitness than insect herbivores (Crawley 1989). Thus, grazers cause similar types of damage to many coexisting species in plant communities. Furthermore, grazers can influence plant evolution indirectly by altering community structure, competitive interactions and abiotic factors such as nutrient availability (McNaughton 1979; Pollard 1992; Olofsson, de Mazancourt & Crawley 2007). These attributes of grazers make them potentially important drivers of plant defence evolution because grazers are expected to cause consistently strong selection on plant morphological traits and tolerance to herbivory (Kotanen & Rosenthal 2000). Although there is a long history of research demonstrating the evolutionary impacts of grazing herbivores on such traits (Kemp 1937; Detling & Painter 1983; McNaughton 1984; Lennartsson, Tuomi & Nilsson 1997; Rotundo & Aguiar 2008), few studies have experimentally examined the short-term evolutionary impacts of generalist grazing herbivores on coexisting plant populations within the same community (Painter, Detling & Steingraeber 1989; Turley *et al.* 2013). By addressing this gap in our knowledge, we will understand how common herbivore-driven evolution is, which traits evolve as defences and whether coexisting plant species respond in similar ways (i.e. convergent evolution) to the same levels and types of damage.

The grasslands of Silwood Park, England, are an ideal system to examine the evolutionary impact of grazing mammalian herbivores on multiple plant species. At this site, rabbits are keystone herbivores with a generalized diet, consuming many forbs, herbaceous species and seedlings of woody species within the plant community. The ecological effects of rabbits have been studied extensively using long-term rabbit exclosures (Crawley 1990; Del-Val & Crawley 2005a), showing that rabbit grazing causes some plant species to increase and others to decrease in abundance (Del-Val & Crawley 2005b). This manipulation also presents the opportunity to study the evolutionary impacts of grazing because plant populations inside and outside rabbit exclosures experience different selection regimes. Our previous work, using the same long-term experiment from Silwood Park, showed that *Rumex acetosa* L. (Polygonaceae) evolved decreased growth rate in the absence of rabbit grazing, but we found no evidence for evolution in chemical defences or tolerance to herbivory (Turley *et al.* 2013). Whether other species in the same community show similar patterns of evolutionary responses is unknown.

Here, we test the hypothesis that grazing mammalian herbivores cause convergent evolutionary changes in plant defences of multiple coexisting species. This was accomplished using a common garden study to test whether the long-term exclusion of rabbits drives contemporary evolution within plant populations of four grassland species. For each species, we asked whether the removal of rabbit grazing from whole communities caused the evolution of: (i) decreased tolerance and (ii) changes in morphology leading to an erect growth form. Answering these questions will allow us to better understand the evolutionary impacts of herbivores on plant

defensive traits and coexisting plant species on a contemporary time-scale.

Materials and methods

STUDY SYSTEM

We conducted a long-term field study at Silwood Park, Imperial College, England, United Kingdom (51.409 N, 0.642 W). Our study site is a species-poor grassland on sandy, acidic soil (National Vegetation Classification: acidic variant of MG6) (Rodwell 1992), where introduced European rabbits (*Oryctolagus cuniculus*) have been present for several thousand years and very abundant since the 1950s (Crawley 1990; Dobson & Crawley 1994). Rabbit grazing alters plant species composition, prevents tree regeneration, affects primary productivity and nutrient dynamics and causes plants to reproduce primarily from clonal vegetative growth rather than seeds (Crawley 1990; Dobson & Crawley 1994; Edwards & Crawley 1999; Olofsson, de Mazancourt & Crawley 2007).

Between 1975 and 2009, we set up 38 rabbit exclosures that ranged in size from 16 to 800 m². These exclosures represent small local plant communities that vary in the amount of time without rabbit grazing, from 4 months to 34 years. Intense rabbit grazing results in short vegetation outside of exclosures and tall vegetation within exclosures (Fig. 1). To study evolution of plant populations following the removal of rabbits, we collected seeds in 2009 from all fruiting plants (typically >10 individuals) of *Anthoxanthum odoratum* (Poaceae), *Festuca rubra* ssp. *rubra* (Poaceae), *Holcus lanatus* (Poaceae) and *Stellaria graminea* (Caryophyllaceae) (see Fig. S1 in Supporting Information) from 23 (age range: 0–34), 16 (age range: 0–21), 23 (age range: 0–21) and 12 (age range: 0–21) rabbit exclosures, respectively. We define a plant population as the plants within a single exclosure. The reduced replication of some populations of species is because we could not find multiple fruiting individuals of all species in every exclosure. We did not collect seeds outside of the exclosures because seeds collected from exclosures created in 2009 are representative of these continuously grazed plant populations.

All of the species studied are common perennials consumed by rabbits at Silwood Park. *Anthoxanthum odoratum* and *H. lanatus* are grasses with a tufted growth form, limited capacity for vegetative reproduction (Grime, Hodgson & Hunt 1988), and they typically increase in abundance with intense rabbit grazing (Crawley 1990;



Fig. 1. Image of a rabbit exclosure at Silwood Park, England. Plant populations outside the rabbit exclosure experience intense rabbit grazing and reproduce mainly vegetatively, whereas plants inside produce flowers and seeds. Photo credit: N. Turley.

Del-Val & Crawley 2005b). In the case of *A. odoratum*, this increase could be a result of being less palatable than other grasses due to its high coumarin content (Grime, Hodgson & Hunt 1988). *Festuca rubra* is a grass with a sod-forming growth form that spreads vegetatively through stolons and rhizomes (Grime, Hodgson & Hunt 1988). It is the dominant species in Silwood Park and in other southern England grasslands (Edwards & Crawley 1999); however, it typically decreases in abundance under intense rabbit grazing (Grime, Hodgson & Hunt 1988; Crawley 1990; Del-Val & Crawley 2004, 2005b). *Stellaria graminea* is a dicotyledonous herb with a slender creeping stock and flowering and non-flowering shoots (Fitter & Peat 1994), and it also typically decreases in abundance under intense rabbit grazing (Crawley 1990).

COMMON GARDEN STUDY

To study the evolutionary impacts of rabbits on plant defences, we established a rooftop common garden at the University of Toronto Mississauga during summer 2012 (Fig. S2). For each species described above, we germinated seeds collected from Silwood Park in sealed Petri dishes on moist filter paper using 0.1% potassium nitrate solution. Seedlings were transferred to 100-mL plastic pots with soil (Pro-mix BX Mycorrhizae, Quakertown, PA, USA) and approximately 0.2 g of slow-release fertilizer (Smartcote, N:P:K, 14:14:14, Brantford, ON, CAN). We started the seedlings in a glasshouse and transferred plants at the three to four true leaf stage to the outdoor rooftop. The location of all plants was completely randomized, and we watered plants twice a day for the duration of the experiment.

The entire experiment consisted of 1183 plants. The number of replicate plants per rabbit exclosure for *A. odoratum*, *F. rubra*, *H. lanatus* and *S. graminea* was 14 ± 9 (mean \pm standard deviation; range: 1–24), 13 ± 7 (range: 4–24), 16 ± 10 (range: 3–24) and 16 ± 7 (range: 7–24) individuals, respectively. Some exclosure populations were represented by fewer plants because of poor germination.

MEASUREMENT OF PLANT TRAITS

We measured five traits that represented morphological, tolerance and avoidance characteristics of the study species. We counted the number of fully expanded leaves on all four study species 24 days after planting. Measurements taken on this day only included plants from the undamaged treatment.

We measured tolerance to rabbit grazing by damaging a subset of plants. We simulated rabbit grazing by removing the above-ground biomass of plants to a height of 1 cm above the soil surface using a razor blade as per Turley *et al.* (2013). For populations with 11 or more replicate plants, we damaged a random half of the plants. For populations with 10 plants or less, we did not impose a damage treatment. Thus, our damage treatment was applied to 12 of the 23 populations of *A. odoratum*, nine of the 16 populations of *F. rubra*, 18 of the 23 populations of *H. lanatus* and 11 of the 12 populations of *S. graminea*. We damaged plants three times: 21, 31 and 41 days after planting to simulate the type of repeated grazing experienced in plant communities at Silwood Park. After 51 days, we harvested the above-ground biomass of damaged and undamaged plants and dried the plant tissue in a drying oven for 72 h at 50 °C. To measure tolerance, we compared the above-ground biomass (tissue collected at final harvest) from damaged and undamaged plants. Tolerance was calculated as follows: (damaged plant biomass – undamaged plant

biomass)/(undamaged plant biomass). We used this relative rather than an absolute measure of tolerance because plants varied in size and our damage treatment was imposed in a relative fashion by removing tissue at a fixed height (Wise & Carr 2008).

Fifty-one days after planting, we measured four morphological traits commonly associated with growth form: culm angle, number of leaves, plant height and plant width (Mahmoud, Grime & Furness 1975; Detling & Painter 1983; Rotundo & Aguiar 2008). In grasses, the culm angle provided a measure of prostrate growth, which we quantified using a protractor as the degrees (0–90°) from the horizontal soil surface to the five most vertical culms. For all four species, we counted the number of fully expanded leaves and measured plant height and width as the tallest vertical or widest horizontal section of the plant, respectively (Fig. S3).

STATISTICAL ANALYSIS

We used linear regressions to test for the evolution of plant traits following the removal of rabbits. For each species, we fit linear models with age of enclosure as a predictor of the average plant trait value per rabbit enclosure (population mean). We infer a significant relationship between age of enclosure and traits of plants grown from these enclosures as evidence for the evolution of plant traits following the removal of rabbits. All models were fitted with the `lm` function of the `stats` package version 2.15.3 in R (R Development Core Team 2012). We also conducted analyses using mixed-effects models implemented with the `lme4` package version 0.999-0 in R, which used all plant data rather than plot-level means. Results for the mixed-effects models were very similar to the linear regressions, so details and results from the former are not presented here but are included as Supporting Information (Table S1). Lastly, we tested for correlations in traits among populations for all four species using Pearson's correlations (r) (Table S2).

Results

Following the exclusion of rabbits for >20 years, we observed an evolutionary change in plant defence traits. We found strong evidence for the evolution of multiple traits in one species (*F. rubra*), weak evidence for the evolution of avoidance in all three grass species, and no evolution in the herb species. The evolution of tolerance and leaf number was idiosyncratic across the four study species. The clearest response was observed in *F. rubra*, where 21 years without rabbit grazing caused a 9% decline in tolerance to herbivory (Fig. 2). The lower tolerance resulted from a 48% decline in final biomass in damaged plants, whereas there was no significant change in final biomass for undamaged plants (Table 1). Similarly, the exclusion of rabbits drove a 26% decline in leaf number in *F. rubra* prior to the damage treatment (Fig. 2). Following the damage treatment, leaf number declined by 51% in *F. rubra*; however, there was no clear change in undamaged populations (Table 1). The absence of rabbit grazing did not result in an evolutionary change in tolerance to herbivory and leaf number in *A. odoratum*, *H. lanatus* or *S. graminea* (Table 1).

Across the three grass species, we found weak evidence for similar evolutionary changes in avoidance traits (Fig. 3). Some species showed increased height/width ratio, whereas others showed increased culm angles (Table 1), which are two methods to capture similar changes in plant growth form (i.e. prostrate growth or erect growth). In the absence of rabbit grazing, height/width ratio increased by 20% in undamaged plants of *H. lanatus* and 41% in damaged plants of *F. rubra*, while there was no evolutionary change in *A.*

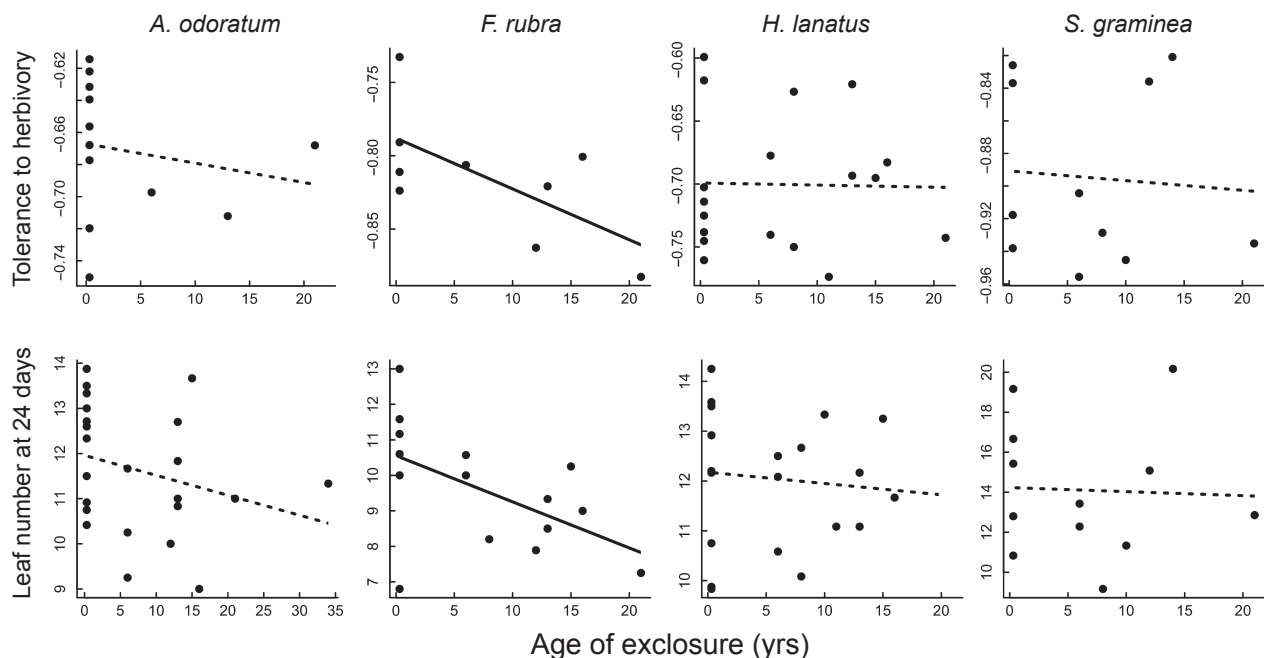


Fig. 2. Tolerance to herbivory and leaf number at 24 days as a function of time since rabbit grazing for all four species. Tolerance is defined as the relative difference in above-ground biomass of damaged and undamaged plants. Graphs with solid best-fit lines show significant ($P < 0.05$) relationships, and dashed lines show non-significant relationships ($P > 0.05$). These figures demonstrate that only *Festuca rubra* showed significant evolutionary responses following rabbit exclusion in these traits.

Table 1. Model results (percentage change effect sizes, *t*-values, degrees of freedom, *P*-values and *R*² values) testing the effects of plot age on plant defence traits of four species. A significant relationship (*P* < 0.05) indicates the evolution of a plant trait following the removal of rabbits. Percentage effect sizes are based on predicted means from the linear regression model at the youngest and oldest enclosure ages present in each data set, and they are interpreted as the percentage change in the plant trait following the removal of rabbits. Positive and negative values indicate an increase or decrease in mean trait values, respectively, following rabbit removal. 'Undamaged' indicates the measurement was taken on an undamaged plant, whereas 'damaged' indicates the measurement was taken on a damaged plant. Bold values indicate responses that are significant (*P* < 0.05)

	<i>A. odoratum</i>			<i>F. rubra</i>			<i>H. lanatus</i>			<i>S. graminea</i>							
	%	<i>t</i>	d.f.	<i>P</i>	<i>R</i> ²	%	<i>t</i>	d.f.	<i>P</i>	<i>R</i> ²	%	<i>t</i>	d.f.	<i>P</i>	<i>R</i> ²		
Tolerance	-0.62	10	0.55	0.04	-9	-2.27	7	0.06	0.42	-0.08	16	0.93	<0.001	-0.23	9	0.83	0.01
Final biomass undamaged	0.78	21	0.45	0.03	-0.90	14	0.38	0.05	0.79	21	0.44	0.03	0.60	10	0.57	0.03	0.03
Final biomass damaged	0.71	10	0.49	0.05	-48	-3.12	7	0.02	0.58	0.47	16	0.64	0.01	-0.40	9	0.70	0.02
Final # leaves undamaged	-0.54	21	0.59	0.01	-1.13	14	0.28	0.08	-0.55	21	0.59	0.01	-0.85	10	0.41	0.07	0.07
Final # leaves damaged	-0.69	10	0.51	0.04	-51	-2.95	7	0.02	0.55	0.27	16	0.79	<0.01	-0.73	9	0.48	0.06
Leaf # at 24 days	-1.34	21	0.19	0.08	-26	-2.44	14	0.03	0.30	-0.58	21	0.57	0.02	-0.13	10	0.90	<0.01
Height/width undamaged	0.10	21	0.92	<0.001	1.55	14	0.14	0.15	20	2.83	21	0.01	0.28	0.40	10	0.70	0.02
Height/width damaged	-0.55	10	0.59	0.03	41	3.07	7	0.02	0.57	-0.29	16	0.77	0.01	0.16	9	0.87	<0.01
Culm angle	40	2.07	21	0.05	0.17	0.65	14	0.52	0.03	1.60	21	0.12	0.11	NA	NA	NA	NA

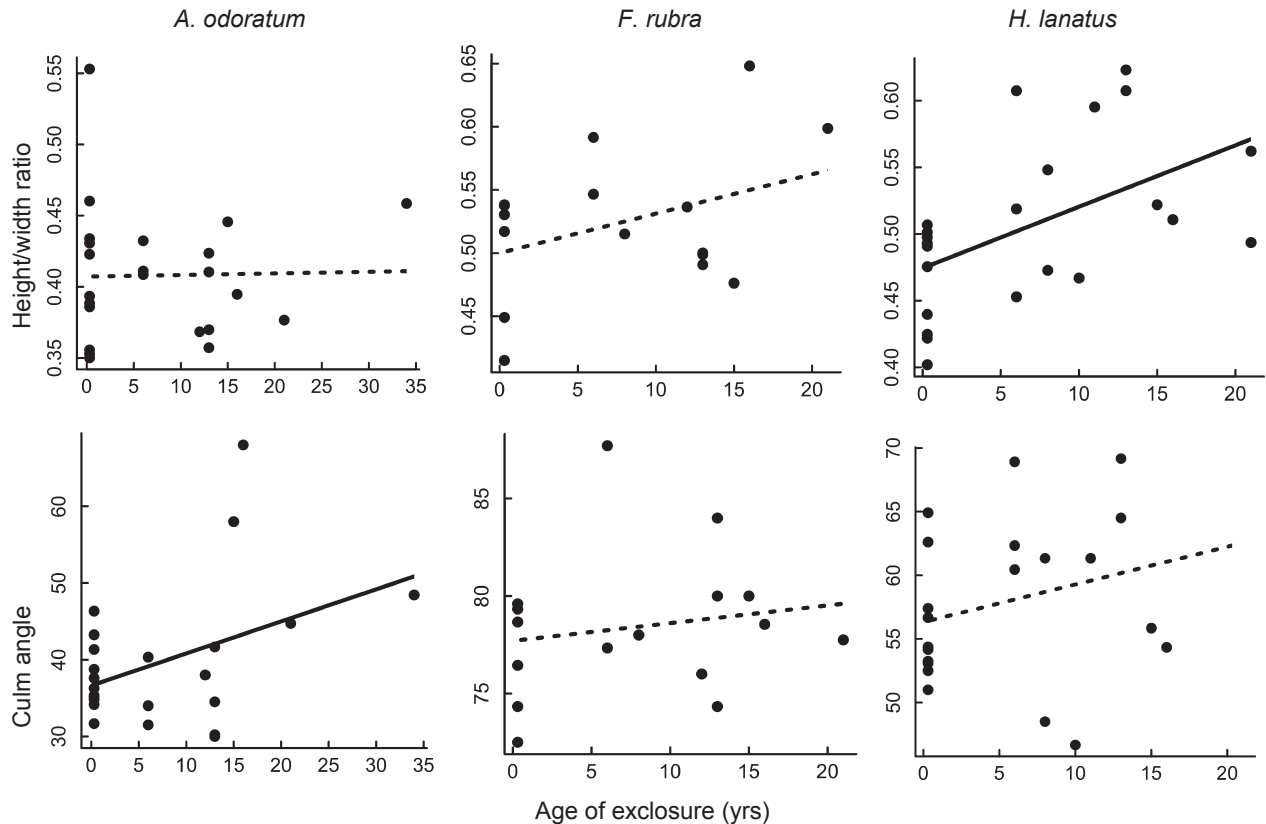


Fig. 3. Avoidance traits as a function of the time since rabbit grazing for undamaged plants of the three grass species. Graphs with solid best-fit lines show significant ($P < 0.05$) relationships, and dashed lines show non-significant relationships ($P > 0.05$). These figures suggest two of three grass species show evidence for evolution of a more erect growth form following the removal of rabbits. Note that *Festuca rubra* showed significant changes in height/width ratio in the undamaged treatments, not shown here (Table 1).

odoratum (Fig. 3, Table 1). In contrast, culm angles increased by 40% in *A. odoratum*, while there was no evolutionary change in *F. rubra* and *H. lanatus*.

We tested for population-level correlations between traits of the four species. For the most part, correlations between traits were intuitive (e.g. positive correlations between width and final biomass and between height and height/width ratio; Table S2). However, we found a negative correlation between final biomass and tolerance in *H. lanatus* ($r = -0.74$, d.f. = 16, $P < 0.001$), but not in the other species (Table S2). We also found no correlation between tolerance to herbivory and leaf number in *F. rubra*, despite the strong evolutionary response of these two traits ($r = 0.09$, d.f. = 7, $P = 0.82$).

We conducted binomial expansion tests (Zar 1996) to determine the probability of observing the number of significant tests reported in Table 1. Across all species, six of 35 tests were significant at $P < 0.05$ (Table 1), a frequency that is unlikely to have occurred by chance (binomial probability: $P = 0.006$). Within *F. rubra*, four of nine tests were significant at $P < 0.05$, and this frequency also has a low probability ($P < 0.001$). By contrast, *A. odoratum* had one of nine significant tests at $P = 0.05$, and *H. lanatus* had one of nine significant tests at $P = 0.01$, which correspond to binomial probabilities of $P = 0.3$ and $P = 0.09$, respectively. Thus, these results support our interpretation of strong evidence of

phenotypic change in *F. rubra* and weak evidence of change in other species. 'Strong evidence' is used here to mean that multiple traits show significant change, and the binomial expansion tests show these results are unlikely due to chance. 'Weak evidence' is used to mean that only a single trait showed a significant change in each species, and the binomial expansion tests show these results could be due to chance.

Discussion

Our study provides a powerful experimental test of the evolutionary effects of excluding an ecologically important herbivore from plant communities. In this system, rabbit grazing impacts plant stature and reproduction, community composition, competitive interactions, ecological succession, and nutrient availability (Crawley 1990, 1997; Del-Val & Crawley 2005a; Olofsson, de Mazancourt & Crawley 2007). Given these large ecological effects, we might expect strong and consistent evolutionary changes across multiple species in this system due to the direct or indirect effects of grazing. We observed clear evolutionary changes in multiple traits for *F. rubra*, weak evidence for the evolution of avoidance in all grass species, and no evolution in an herb species, *S. graminea*. Also, a previous study conducted with this system observed the evolution of a defence trait using a different species

(Turley *et al.* 2013). Thus, twenty-plus years of rabbit exclusion can drive evolutionary change in plant defence and morphological traits, but this is not consistent across species within a community. This suggests that herbivore-driven evolution and the evolutionary impacts of herbivores are varied and difficult to predict based on their ecological importance (Agrawal, Lau & Hamback 2006).

GENETIC VERSUS TRANSGENERATIONAL EFFECTS

All of the study species had ample opportunity for changes in allele or genotype frequency during the time period of our experiment, even given their perennial life history. Species can exhibit changes in allele or genotype frequency through seed recruitment. In the grasslands of Silwood Park, recruitment from seed is relatively uncommon, but not absent, in undisturbed areas (Crawley 1990). Moreover, molehills and rabbit scrapes can make up 2–3% of the soil surface each year, and these disturbed areas are rapidly colonized by seed rain from surrounding plants (Edwards & Crawley 1999). Although there was no disturbance by rabbits in our enclosure plots, disturbance by moles provided ample opportunity for new recruits from seeds in plots during the experiment. In addition to recruitment from seed, vegetative reproduction (e.g. rhizomes, stolons, expansive vegetative tufts) is also common in this system and provides an opportunity for adaptive evolution via clonal lineage selection. Across multiple blocks, new recruits from vegetative reproduction were 1.3–22.3 times more abundant than recruits from seed, and all four study species showed capacity for vegetative reproduction in both grazed and ungrazed plots (Crawley 1990). Thus, even if reproduction in this system was only vegetative (i.e. clonal), evolution could still occur through lineage sorting, causing changes in the relative abundance of different genotypes through time. Finally, a number of studies in similar systems (British grasslands) have shown rapid genetic differentiation of *A. odoratum* over spatial and temporal scales similar to ours (Antonovics & Bradshaw 1970; Snaydon & Davies 1972; Silvertown *et al.* 2005).

Although there was ample opportunity for genetic change within populations and we feel genetic evolution is the most likely explanation for the observed phenotypic changes, we cannot conclusively rule out the possibility that the observed long-term changes in phenotypes were due to transgenerational effects (i.e. maternal effects or epigenetics). Maternal effects mediated by allocation to seed resources are an unlikely explanation because these effects are typically short-lived and thus are not expected to persist through the course of our 7-week common garden experiment (Stanton 1984; Weiner *et al.* 1997; Galloway 2001; Agrawal 2002). In contrast, epigenetic changes are a possible explanation because they are difficult to distinguish from classical genetic evolution, and in some cases, they may be functionally similar if epigenetic modifications to the genome are stably inherited (Bossdorf, Richards & Pigliucci 2008; Weigel & Colot 2012). We know that the parental biotic and abiotic environment can influence an offspring's physiology (Rasmann *et al.* 2012), resistance

to herbivores (Agrawal 2002), life-history strategy (Galloway 2001; Lin & Galloway 2010) and morphology (Cubas, Vincent & Coen 1999; Johannes *et al.* 2009). However, few studies have investigated the ecological determinants of both genetic and epigenetic changes in natural populations. There is especially little known about whether long-term epigenetic changes can be associated with long-term adaptive phenotypic evolution and how important such changes might be relative to genetic changes (Bossdorf, Richards & Pigliucci 2008; Weigel & Colot 2012). A notable exception is a recent long-term study by Herrera & Bazaga (2011) on the perennial herb *Viola cazorlensis*. They found that intensity of mammalian grazing is an important predictor of both genetic (measured using AFLP markers) and epigenetic differences (measured as patterns of DNA methylation) among individuals. It is uncertain whether epigenetic changes influence levels of damage or *vice versa*, but this study suggests that grazing can lead to epigenetic changes in plant populations in the field. Thus, it is possible that some of our observed changes could be due to epigenetic effects or a combination of genetic and epigenetic effects. But for simplicity, hereafter, we call all observed changes 'evolution'.

EVOLUTION OF TOLERANCE

In general, we expect tolerance to increase in the presence of herbivores in order to reduce the negative fitness effects of herbivory (Strauss & Agrawal 1999) and to decrease in the absence of herbivores, assuming a cost to tolerance (Strauss *et al.* 2002; Núñez-Farfán, Fornoni & Valverde 2007). Our results provide only partial support for this prediction because the exclusion of rabbits caused the evolutionary loss of tolerance to herbivory in just one of the four study species, *F. rubra* (Fig. 2). Several other studies have manipulated herbivory and measured subsequent evolution in tolerance. Similar to our findings, these studies also show inconsistent evolutionary responses in tolerance to herbivory. Some studies found that the exclusion of herbivores caused a decrease in tolerance (Detling & Painter 1983; Painter, Detling & Steingraeber 1989; Lennartsson, Tuomi & Nilsson 1997), whereas other studies found no change in tolerance (Jaindl *et al.* 1994; Loreti, Oesterheld & Sala 2001; Turley *et al.* 2013).

Variable evolutionary responses in tolerance following removal of herbivores could be due to multiple factors. First, the focal herbivores might not select on tolerance traits, and thus no evolution is expected to occur. Secondly, tolerance might confer little cost to plants in the absence of herbivores such that high tolerance can be maintained without consistent grazing. Thirdly, traits that confer tolerance often serve multiple functions, and selection by other environmental stressors could be sufficient to maintain high tolerance (Stowe *et al.* 2000; Agrawal, Conner & Stinchcombe 2004; Núñez-Farfán, Fornoni & Valverde 2007). Lastly, genetic or selective constraints could prevent the evolution of decreased tolerance (van der Meijden, Wijn & Verkaar 1988; Arnold 1992). For example, in this study, we found a strong negative correlation

at the population level between final biomass (i.e. growth rate) and tolerance in *H. lanatus* (Table S2). Evolution in growth rate, as seen in our previous study using *Rumex acetosa* from this system (Turley *et al.* 2013), coupled with genetic correlations between growth and tolerance could prevent the expected evolutionary changes in tolerance (Arnold 1992). Distinguishing between all these factors is difficult, but advances in this area would help build our predictive understanding of the evolution of tolerance (Fornoni 2011).

Tolerance may also evolve as part of a suite of traits that are adaptive under environmental conditions with and without herbivores. In addition to the evolution of tolerance, we found that in the absence of rabbit grazing, *F. rubra* produced fewer leaves. Although mechanisms affecting the evolution of tolerance are not well understood (Stowe *et al.* 2000; Schwachtje *et al.* 2006), a growth form with smaller and more abundant leaves could allow *F. rubra* to be more tolerant in the presence of rabbit grazing. McNaughton (1984) suggested that grazing herbivores cause grasses to evolve dense, short and abundant leaves because it prevents apical meristems from being damaged. Our results are consistent with this although we see no correlation between leaf number and tolerance to herbivory at the population level (Table S2). This suggests changes in leaf number did not influence a plant's tolerance but evolved independently because it was adaptive for some other reason.

EVOLUTION OF AVOIDANCE STRATEGIES

We found evidence that exclusion of rabbits causes changes in avoidance traits in all three grass species in our study, although these patterns were relatively weak. Height/width ratio increased following rabbit exclusion in *H. lanatus* and *F. rubra* in our undamaged and damaged treatments, respectively (Table 1). Similarly, in *A. odoratum*, we observed increases in a different measure of growth form, culm angle, but this result should be interpreted with caution given the relatively weak pattern (Fig. 3, Table 1). While these results are not definitive, they do suggest that grass species evolve to be more prostrate in the presence of rabbits and more erect in their absence. This pattern is consistent with other studies that manipulate herbivore presence/absence and measure evolutionary response in plant populations (Painter, Detling & Steingraeber 1989; Rotundo & Aguiar 2008). The leading hypothesis is that prostrate growth is an adaptation to grazing because short and low plants have a smaller percentage of tissue removed by grazing herbivores (Kemp 1937; McNaughton 1984). However, Kotanen & Bergelson (2000) tested this hypothesis directly and found no support that prostrate growth forms are better adapted to grazing herbivores. An alternative hypothesis is that growing low and close to the ground is not an adaptation to lessen the amount of herbivore damage but rather to increase the ability of grasses to reproduce vegetatively by facilitating spread through stolons and rhizomes. Because plants have no opportunity to reproduce by seed under intense grazing, increased capacity to reproduce vegetatively could be adaptive. The evolution of an erect growth

form following the removal of rabbits likely occurs due to changes in the competitive environment (Mahmoud, Grime & Furness 1975). In grazed environments, the vegetation is consistently clipped close to the ground, whereas in rabbit enclosures, the vegetation is tall and dense (Fig. 1). We speculate that genotypes with an erect growth form increased in frequency following removal of rabbits because they could capture light more effectively in ungrazed environments (Dudley & Schmitt 1996). Although the evolutionary roles of competition and herbivory are expected to act in concert (Herms & Mattson 1992; Agrawal 2004; Agrawal, Lau & Hamback 2006), more studies are needed to support this assertion in our system.

EVOLUTIONARY RESPONSES AMONG SPECIES

There are numerous factors which could explain the varied and lack of evolutionary responses among species in this experiment. Variation in plant life histories and trait values could result in different patterns of selection, even under similar ecological conditions. We still have limited understanding if similar patterns of herbivory on multiple species can drive convergent evolution of plant defence syndromes, especially in a microevolutionary context (Feeny 1976; Kursar & Coley 2003; Agrawal & Fishbein 2006). We found some evidence for convergence in avoidance traits across multiple species; thus, for this trait, the presence/absence of rabbit grazing seemed to result in similar evolutionary responses among species. In other traits, however, we did not see consistent patterns across species.

Community complexity could also be an important factor leading to variable evolutionary responses among species (Miller & Travis 1996; Strauss 2014). For example, the removal of rabbits can alter community composition, competition among plants, the frequency of disturbance and nutrient dynamics (among other factors) (Edwards & Crawley 1999; Del-Val & Crawley 2005a; Olofsson, de Mazancourt & Crawley 2007). These complex direct and indirect effects of herbivores could all potentially be important agents of selection which can make it very difficult to predict evolutionary outcomes in plant–herbivore interactions in a community context (Agrawal, Lau & Hamback 2006).

Finally, population size is another potentially important factor influencing the frequency and rate of evolutionary change. Evolutionary responses to selection can be constrained in small populations where the effects of genetic drift are stronger compared with large populations (Wright 1931; Leimu & Fischer 2008). Percentage cover data from a separate field experiment at Silwood Park that included both fenced (22 years of rabbit removal) and unfenced plots suggest that population size may be important (M. J. Crawley, unpubl. data). These data show that *F. rubra* had the greatest percentage cover (fenced: 32.99%; unfenced 28.72%), followed by *H. lanatus* (fenced: 6.34%; unfenced: 14.35%), *A. odoratum* (fenced: 4.85%; unfenced: 2.61%) and *S. graminea* (fenced: 0.42%; unfenced: 0.96%). The rank order of expected population sizes (i.e. *F. rubra* » *H. lanatus* > *A. odoratum* > *S. graminea*) corresponds closely

to the amount of evolutionary change we observed for each species (Table 1). This supports theoretical predictions and empirical evidence that population size is an important predictor for the ability of plants to adapt over small temporal and spatial scales (Wright 1931; Linhart & Grant 1996; Leimu & Fischer 2008).

Conclusions

Our study provides a rare empirical test of the evolutionary effects of the presence/absence of an herbivore on multiple plant species. Although we found some evolutionary changes in three of the four species, the evidence was strong for the evolution of tolerance in only one species, while the evidence was weak for convergent evolution of prostrate growth in the three grass species. We observed the strongest evidence for evolutionary change in *F. rubra*, which is the most abundant species in this system. This suggests that population size may be an important predictor of the evolutionary response to the presence/absence of herbivores. As our manipulations were conducted on whole communities, evolutionary responses, or lack thereof, are a consequence of many direct and indirect effects of herbivores which may constrain evolution and make it difficult to predict evolutionary responses. Future studies are needed to explore the evolutionary constraints of defence traits among species from the same community and tease apart the relative strengths of the direct and indirect effects of herbivory in driving evolution of plant defences.

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Data accessibility

Trait measurements collected from plants in our common garden experiment. Data available from the Dryad Digital Repository (Didiano *et al.* 2014).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Image of our four study species after 51 days of planting.

Figure S2. Image of our common garden study conducted at the University of Toronto Mississauga in summer 2012.

Figure S3. Image illustrating the culm angle and height/width ratio measurement.

Table S1. Results of mixed-effects models testing for the evolution of multiple traits following the removal of rabbits for all four plant species.

Table S2. Pearson's correlations between traits among populations for all four species.