ARTICLE

Reducing herbicide input and optimizing spray method can minimize nontarget impacts on native grassland plant species

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Abstract

Invasive plants threaten biodiversity worldwide and effective management must control the target invader while conserving biodiversity. Herbicide is often used to control invasive plants, but potential negative impacts on biodiversity have led to spot spraying being recommended over boom spraying to minimize the exposure of nontarget species to chemicals. We examined the influence of herbicide application methods on off-target plant communities in threatened temperate grasslands of southeastern Australia, where spraying with the broadleaf herbicide fluroxypr is commonly used to control St. John's wort, *Hypericum perforatum* L. It is well established that fluroxypr effectively controls *H. perforatum* but few studies have examined its impact on native forbs. A spray drift experiment using water-sensitive cards indicated that ground surface coverage was higher for spot spraying (91%–99%) than for boom spraying (5%–31%). We established a replicated, 3-year, before-after-control-impact experiment across 48 1-m^2 quadrats to determine how three herbicide application methods (spot spray, fine boom and coarse boom) affected nontarget native forbs, the group most likely to be affected by broadleaf herbicides. This experiment was conducted in grasslands where *H. perforatum* was almost absent, so responses would reflect the direct impacts of the chemical, rather than structural changes resulting from removal of the target invader. Spot spraying decreased the probability of occurrence of native leguminous forbs, while increasing the occurrence of exotic leguminous forbs and the richness of all exotic species and exotic annual forbs. Spot spraying reduced the occurrence of the native *Desmodium varians* and the abundance of the native *Chrysocephalum apiculatum*. During this 3-year study, native species appeared to be impacted either directly by fluroxypr or indirectly by increased competition with exotic species. Where herbicide application is deemed crucial in these grasslands, we recommend boom spraying when *H. perforatum* density is moderate to high. Spot spraying should only be used

when the density of *H. perforatum* is very low. Given the regional variation in *H. perforatum* density, the spatial scale of invasion, soil depth, and conservation values, we present a decision tree to assist managers in evaluating the costs and benefits of chemical control, indicating situations where alternative or modified methods could be used.

INTRODUCTION

Invasive plants threaten biodiversity worldwide by displacing resident individuals (Thiele et al., 2010), limiting colonization rates of new individuals (Yurkonis et al., 2005), changing plant community dynamics (Catford et al., 2019), and altering ecosystem processes, including disturbance regimes (e.g., grazing and fire; Fusco et al., 2019) and nutrient cycling (Ahmad et al., 2021; Broadbent et al., 2017; Ehrenfeld, 2003). Invasion of exotic species can alter life-history trade-offs (such as competition vs. colonization, Catford et al., 2018), a process which might underlie invasion-associated extinction risk globally (Baider & Florens, 2011; Briggs & Leigh, 1996; Dangremond et al., 2010; Downey & Richardson, 2016; Pyšek et al., 2017). Invasion-associated declines in biodiversity can reduce ecosystem productivity, resilience and stability (Vetter et al., 2020). Managing invasive species is therefore critical to conserving biodiversity and ecosystem function (Ricciardi et al., 2021; Veldman et al., 2015).

Globally, billions of dollars are spent every year on controlling and managing invasive species (Cuthbert et al., 2021; Gaba et al., 2016; Pimentel et al., 2005; Setterfield et al., 2013; Watari et al., 2021), a cost that is rising continuously (Diagne et al., 2021). The overarching objectives of invasive plant control are generally to conserve biodiversity (Reid et al., 2009; UNEP, 2011), but outcomes are more commonly measured by the impact on the cover and abundance of the target invasive plant, assuming this will have positive biodiversity outcomes (Downey et al., 2009; Kettenring & Adams, 2011; Zavaleta et al., 2001). While there are examples of weed management programs having positive biodiversity outcomes (e.g., Flory, 2010; Lindenmayer et al., 2017; Peterson et al., 2020; Rohal et al., 2019), monitoring biodiversity is far from routine in invasive species control programs, usually because of lack of time and money (D'Antonio & Meyerson, 2002; Downey et al., 2009; Kettenring & Adams, 2011; King & Downey, 2008; Reid et al., 2009). In some circumstances, the localized effects of invasive plant control on native species have been

neutral (O'Loughlin et al., 2019; Rice et al., 1997) or negative (Rinella et al., 2009; Skurski, 2013). Other studies have found that controlling target invaders can facilitate the invasion of the same (secondary invasion) or a different invasive species (Peterson et al., 2020; Reid et al., 2009) because of reduced competition and increased resource availability (Pearson et al., 2016). Given the wide range of responses across different plant communities, it is clear that invasive species control should not be measured by the impact of the target invader alone (Prior et al., 2018). Monitoring resident plant communities during weed control must be planned for, implemented, appropriately funded, and quantitatively reported if management programs are to meet their goals of conserving biodiversity.

Most land managers consider herbicide to be the most economical and effective method for controlling invasive plants, and it is the most commonly used control method in high-income countries worldwide (Kettenring & Adams, 2011; Van Bruggen et al., 2018; Weidlich et al., 2020). In Canada, for example, more than 1 million ha of wildlands were sprayed with herbicide in the period 2007–2011 (Wagner et al., 2017). Although some studies have found largely neutral effects of herbicide on ecosystem function (e.g., decomposition and nutrient cycling, Hagner et al., 2019) several negative impacts have also been found. Herbicide use has been associated with declines in native plant species cover, richness and community composition (Guido & Pillar, 2017; Peterson et al., 2020; Qi et al., 2020; Skurski, 2013). Herbicide can alter phenology and negatively impact the reproduction rates of native species (Rokich et al., 2009; Wagner & Nelson, 2014), even when effects on biomass and vegetative traits are not strong (Carpenter et al., 2020; Crone et al., 2009; Schmitz et al., 2014). Such effects on plant reproduction can carry over to the F1 generation (Qi et al., 2017). Forbs are particularly at risk from herbicides that target broadleaf groups (Crone et al., 2009) while native shrubs and grasses can be threatened by broad-spectrum herbicides (Matarczyk et al., 2002; Power et al., 2013; Rokich et al., 2009). Herbicide can also have negative impacts on soil microbial communities (Druille et al., 2016),

pollination (Dupont et al., 2018; Motta et al., 2018), and terrestrial and aquatic animal communities (Haughton et al., 1999; Relyea, 2005; Rumschlag et al., 2020; Saska et al., 2016; Van Bruggen et al., 2018). The ecological effects of herbicide use, therefore, must be understood so that the risks can be weighed against the impacts of the weeds themselves (Gaba et al., 2016; Skurski, 2013).

The herbicide application method is considered important when managing native ecosystems as it influences spray drift to nontreatment areas that can negatively affect biodiversity and ecosystem function (Dupont et al., 2018; Florencia et al., 2017; Gove et al., 2007; Strandberg et al., 2021). It is widely assumed that selective spot spraying (treating individual plants using a manually operated spray lance) will have fewer off-target impacts on native species than broadcast or boom spraying (treating patches of several plants with a vehicle-mounted boom spray; GeFellers et al., 2020; Hunt, 2017; Rokich et al., 2009) and some research supports this hypothesis. For example, Power et al. (2013) found that automated spot spraying had fewer nontarget impacts on native species than manual broadcast or spot spraying (although it was not effective in reducing the target weed). Another study found that broadcast spraying was more effective in heavily invaded systems while spot spraying was more effective in predominantly native systems (Nyamai et al., 2011). Spray droplet size affects herbicide uptake in target weeds, with larger, more concentrated, droplets increasing efficacy over several fine droplets (Cranmer & Linscott, 1991; Feng et al., 2003; Ramsdale et al., 2003). Large droplets might limit horizontal spray drift to nontreatment areas, while fine droplets may limit vertical spray drift, but few studies have quantified the impacts of herbicide spray method and droplet size on native plant communities (Cederlund, 2017).

In this study, we examined the short-term (3-year) influence of herbicide application on off-target plant communities in threatened temperate grasslands of southeastern Australia. One of the most difficult-to-manage invasive plants in this system is *Hypericum perforatum* L. (St. John's wort) which can form dense infestations in highly diverse native grasslands (Smith et al., 2018). Spraying with the broadleaf herbicide fluroxypr is commonly used by land managers to control *H. perforatum*, with the aim of conserving biodiversity. It is well established, both in the literature (e.g., Ainsworth & Mahr, 2001; Campbell & Nicol, 1997) and through decades of on-ground experience in our study system, that fluroxypr effectively controls *H. perforatum*, even at the low levels typically found in spray drift (Kleijn & Snoeijing, 1997). We have observed, however, that herbicide application appears to be associated with a decline in the presence and cover of native forbs. Few, if any, tests of this observation exist in the literature.

Therefore, we set out to investigate the impact of fluroxypr application on native grassland species and develop weed management guidelines that have positive biodiversity outcomes, while also effectively controlling the target invader.

We first conducted a spray drift experiment in grasslands invaded by *H. perforatum* to address the question:

1. How do three commonly used herbicide application methods—spot, fine-boom, or coarse-boom spraying—vary in spray distribution and droplet density?

We then conducted a nontarget impacts experiment in grasslands rich in native forbs where *H. perforatum* was almost absent to address the questions:

2. What is the influence of spot and boom spraying on the richness and diversity of nontarget resident plant species?
3. What is the influence of spot and boom spraying on the occurrence and abundance of individual native forb species?

We focused on biodiversity metrics used by government agencies to monitor grassland quality (e.g., "indicator" species richness) and on functional groups that provide insight into the mechanism underlying observed responses (e.g., legumes involved in N cycling). Finally, we constructed a decision tree combining the outcomes of this study with previously published literature and our field experience in managing grasslands to make specific recommendations about controlling *H. perforatum* under different circumstances. Ultimately, we aimed to provide evidence-based guidelines for land managers under urgent pressure to conserve grassland biodiversity through practical and affordable weed management techniques.

MATERIALS AND METHODS

Study region and target invader

The study was undertaken in three grassland reserves in the Australian Capital Territory (ACT), near the capital city of Canberra: Kama Nature Reserve, Jerrabomberra West Nature Reserve and Mulanggari Grasslands Nature Reserve (hereafter Kama, Jerrabomberra, and Mulanggari, respectively). The region has a cool temperate climate, with mean temperatures ranging from 13°C to 28°C in summer to 0–11°C in winter (Australian Government Bureau of Meteorology). Mean annual precipitation is 629 mm but is highly variable and rainfall is generally distributed throughout the year. The ecological community is classified as Natural Temperate Grassland and is listed as

threatened at the regional level (ACT Government *Nature Conservation Act 2014*) and critically endangered at the national level (Australian Government *Environment Protection and Biodiversity Conservation Act 1999*). Approximately 95% of Natural Temperate Grassland in Australia has been cleared or degraded since European colonization (Keith, 2004). Only small fragments (25–140 ha) remain in an urban and agricultural matrix (ACT Government, 2005; Williams & Morgan, 2015). Common native grasses include *Themeda*, *Austrostipa*, *Rytidosperma*, *Poa*, and *Bothriochloa* species and common exotic grasses include *Avena*, *Phalaris*, *Bromus*, *Aira*, and *Vulpia* species. There are two dominant native perennial grass types in the study region: *Themeda triandra* (C4) dominated and *Austrostipa/Rytidosperma* spp. (C3) dominated.

H. perforatum is native to Europe and, following its introduction in the mid-1800s for medicinal and ornamental purposes, it became widespread and invasive in temperate ecosystems of southeast Australia, including in our study reserves (Harris & Gill, 1997). The species is classed as invasive in its nonnative range (Buckley et al., 2003) because it reproduces prolifically and spreads over large areas (Richardson et al., 2000). We followed this definition of "invasive" to refer to *H. perforatum* and other plant species with this characteristic. We used the more general term "exotic" to refer to nonnative plant taxa introduced by humans (Richardson et al., 2000), without necessarily indicating impact or spread. The species has a lifespan of 6–7 years and can reproduce sexually or asexually through apomyxis and vegetative suckering (Buckley et al., 2003). Viable seeds can remain dormant in the soil for up to 20 years (Groves, 1997). In its nonnative North American range, *H. perforatum* has high genetic diversity (Maron et al., 2004) and can establish across broad environmental gradients without the need to "match" conditions in its native range (Maron, 2006). In New Zealand, populations have higher density, fecundity, vegetative reproduction and germination rates compared with native European counterparts (Beckmann et al., 2009, 2011).

In the ACT, *H. perforatum* is listed as a declared pest (ACT Government, 2015) mainly due to its status as an agricultural weed (being toxic to stock and competing with pasture species; Groves, 1997). Management agencies are legally required to actively prevent infestations from spreading to neighboring premises. Although it is believed to outcompete native species and threaten biodiversity, there is surprisingly little evidence to support this assumption (Cullen et al., 1997) and its impact is currently unknown. Despite this, substantial resources have been dedicated to controlling *H. perforatum* in nature reserves that are managed primarily for biodiversity conservation. For example, in 2016–2017, 2673 ha of *H. perforatum* were sprayed across nature reserves in the ACT. *H. perforatum* is typically controlled through spot or boom spraying using the narrow-spectrum broadleaf herbicide fluroxypyr. This chemical does not kill grasses (Campbell & Nicol, 1997) and is assumed to have minimal impacts on native forb species, but no studies have tested these assumptions. In 1997, a workshop of experts called for urgent research on the impacts of *H. perforatum* control methods on native species (Cullen et al., 1997). Some studies have examined nontarget impacts of biological control of *H. perforatum* (e.g., Willis et al., 2003), but we are unaware of any studies that have tackled this issue for chemical control, in our study region or in grasslands generally. This knowledge gap makes it impossible to effectively determine the costs and benefits of the chemical control of *H. perforatum*. There is growing concern among managers that controlling *H. perforatum* with herbicide is doing more damage than the species itself.

Study design overview

Grasslands in our study area are broadly classified as Natural Temperate Grassland but there is variation across the region in the composition of dominant grasses (Smith et al., 2018), reflecting variation in soil depth, nutrient status, and disturbance regime. This variation drives differences in grassland physical structure (Morgan & Salmon, 2020) and biodiversity (Smith et al., 2018). The composition of dominant grasses therefore influenced which reserves we targeted for the two experiments. The spray drift experiment (Question 1) was conducted in Kama (10 km west of Canberra), where the C4 grass *T. triandra* is dominant and *H. perforatum* is widespread with variable density (Smith et al., 2018). This allowed us to compare spray drift for each herbicide application method in areas without *H. perforatum* and areas where it was present at moderate-to-high densities, approximating scenarios frequently encountered by land managers. If the density of *H. perforatum* influenced the amount of chemical that reached the ground (and therefore nontarget species), guidelines for spraying could be modified depending on the target invader's cover.

The nontarget impacts experiment (Questions 2 and 3) was examined at two reserves where *H. perforatum* was rare or absent: Jerrabomberra and Mulanggari, south and north of Canberra, respectively, and separated by ~20 km. Our primary goal was not to demonstrate the chemical's effectiveness (which is already well established), but rather to determine its impact on nontarget species. If we conducted this experiment where *H. perforatum* was common, we would have been unable

separate the effects of changing physical structure from killing the invader (e.g., changes in light, space and nutrient availability) from the primary effects of the chemical. Thus, working where *H. perforatum* was rare or absent allowed us to examine herbicide effects on resident communities without results being confounded by structural changes or previous spray history.

Both reserves are dominated by C3 grasses, chiefly *Austrostipa* and *Rytidosperma* species, but they differ in species composition. *Themeda* is virtually absent from Jerrabomberra (recorded in only one plot in this study), while it is common at Mulanggari (mean percentage cover per sampling quadrat = 7%, range 0%–50%). *Themeda* typically occurs on deep, low-nutrient soils and is often used as an indicator of grassland quality as it declines in abundance with livestock overgrazing (Dorrough et al., 2004), nutrient enrichment (Groves et al., 2003) and fire frequencies that are low (>6 years; Morgan & Lunt, 1999) or very high (biennial; Prober et al., 2007). We previously found that *Themeda* grasslands had higher species richness of some plant functional groups (Smith et al., 2018). Herbicide effects could therefore differ between these grasslands because they have different initial plant compositions. Leaf litter cover was also higher in *Themeda* grasslands, compared with C3 grassland (Smith et al., 2018), which we anticipated could influence the amount of herbicide that reached the ground during spraying. Thus, we treated the two reserves not as replicates but as tests of herbicide effects in different grassland subtypes, which was reflected in our analysis (described below).

Spray drift experiment

To quantify vertical spray drift for the three herbicide application methods (Question 1), we used water-sensitive cards that stained on contact with liquid (Appendix S1: Figure S1). In November 2017, we deployed 30 cards at Kama: 15 in areas with no *H. perforatum* and 15 where *H. perforatum* was present at moderate-to-high densities (i.e., density was a two-level factor). The distance between the two weed-density areas was >100 m. Cards within density levels were separated by ~10 m, over a 5000 m² area, and spray treatments were assigned randomly to cards. Cards were placed ~20 cm above ground on a piece of dowel to correspond with the height that native forb vegetation typically reaches. Cards in the moderate to high-density treatment were consistently positioned at approximately the same distance below the canopy of *H. perforatum*. For each weed-density level, the 15 cards were assigned one of three spray treatments, resulting in five replicates per treatment. For each spray treatment we

used Starane Advanced (fluroxypyr) with 500 ml uptake oil per 100 L of water. The treatments were: (1) spot spraying with a vehicle-mounted quick spray unit and spray lance (rate = 300 mL/100 L water), (2) vehicle-mounted boom spraying (fine droplet) with a twin-bodied nozzle at 60° (rate = 1.8 L/ha, water 100 L/ha) and (3) vehicle-mounted boom spraying (coarse droplet) with a flat pen nozzle at 90° (rate = 1.8 L/ha, water 75 L/ha). Treatments were applied on a windless day to minimize horizontal spray drift. Under both *H. perforatum* density levels, spraying was applied at the same rate, and from the same height above ground (~60 cm for spot spraying and 100 cm for boom spraying). The spray treatments were applied randomly so that no plant functional groups were targeted under either density level. After application, we scanned the cards and used the select function in Adobe Photoshop to calculate the proportion of the card stained by herbicide within a standardized area (Appendix S1: Figure S1).

Nontarget impacts experiment

To test the effects of herbicide on native plant diversity and individual forb species (Questions 2 and 3) we established a before-after-control-impact experiment at Jerrabomberra and Mulanggari. At each reserve, we established eight replicate plots (each ~400 m²), separated by a minimum of 100 m (range 106–387 m). Three permanently marked 1-m² treatment quadrats (control, spot spray and boom spray) were randomly assigned within each of the eight plots, giving 48 quadrats across the two reserves. Quadrats were spaced at least 8 m apart within plots (range 8–14 m) to ensure treatments were spatially separated. Given our interest in fluroxypyr effects on native forbs, the location of the quadrats was chosen to include at least one (and typically four, median across 48 quadrats = 4) species from the following: *Chrysocephalum apiculatum* (Asteraceae), *Eryngium ovinum* (Apiacea), *Tricoryne elatior* (Asphodelaceae), and *Desmodium varians* and/or *Glycine tabacina* (Fabaceae). Other native forbs commonly present in plots included *Lomandra filiformis coriacea*, *Plantago varia*, and *Triptilodiscus pygmaeus*. To the best of our ability, we aimed to keep conditions across quadrats within plots as visually similar as possible, in terms of dominant species, cover of bare ground, and drainage.

Two experienced botanists surveyed vegetation in all quadrats between 2 and 20 November 2017 (spring) before treatment application. The number of individuals of all plant taxa observed within each quadrat were counted (abundance) and assigned a cover score to the nearest 10%. Most taxa (79%) were identified to species

level following NSW Flora Online (http://plantnet.rbgsyd.nsw.gov.au). Twenty-five taxa were difficult to identify and were either assigned to a genus (e.g., *Avena*, *Aira*, *Conyza*, and *Vulpia*) or to three different morphospecies (*Rytidosperma*).

Following the initial plant survey, the herbicide Starane Advanced (fluroxypyr) with 500 ml uptake oil per 100 L water was applied to the treatment quadrats, including buffers, on 18 December and 19 December 2017. Treatment 1 (spot spray) was applied with a vehicle-mounted quick spray unit (pressure = 15 kPA, rate = 300 mL/100 L water). Treatment 2 (boom spray, fine droplet) was applied with a vehicle-mounted twin-bodied nozzle at 60° (rate = 1.8 L/ha and 75 L water/ha). No herbicide was applied to control quadrats and a screen was placed between control and treatment quadrats to reduce the risk of horizontal spray drift. All quadrats were resurveyed between 5 November and 16 November 2018 and 14 November to 23 November 2019 by one of the botanists involved in the initial survey, following the pretreatment survey procedure.

Functional group classification

Our study focused on herbicide effects on native forbs, but a reduction in forbs following fluroxypyr application can drive indirect changes in plant community composition. Thus, we included a suite of functional groups in our analysis to understand the community-level changes to herbicide application (Table 1). Grasses can respond positively to a reduction in forbs following fluroxypyr application (Kleijn & Snoeijing, 1997; Lodge et al., 1994) and we anticipated that C3 and C4 grasses might respond differently given the early summer application of herbicide. Annual forbs that, in our study system, are predominantly exotic (79%), are known to be strong early invaders (Byun et al., 2013; Galland et al., 2019), especially those which can fix nitrogen (Catford et al., 2019). These might therefore have a competitive advantage if predominantly perennial native forbs are reduced. Perennial species, conversely, have slower leaf economics (Reich, 2014) and are likely to have a stronger competitive ability in the longer term (e.g., >5–10 years, Catford et al., 2019). We thus predicted that they would respond weakly or not at all to a short-term reduction in native forbs.

We classified each taxon into categories based on origin (native/exotic), lifespan (annual/perennial), growth form (forb, grass, shrub, sedge, rush), photosynthetic pathway for grasses (C3/C4) and ability to fix nitrogen (legume/nonlegume; Table 1; Díaz et al., 2007; Smith et al., 2018). In addition to these categories widely used

T A B L E 1 Twenty-seven functional groups of plant taxa used to assess effects of herbicide on nontarget plant communities in Natural Temperate Grasslands of southeastern Australia.

Functional group	Levels within group	No. species
All species	All species	119
Grassland quality monitoring	Indicator A	24
	Indicator B	14
	Indicator X/Y	10
	Indicator Z	31
	Common/increaser	40
Origin	Native	78
	Exotic	41
Growth form	Sedge/rush	6
	C3 grass[a]	25
Origin × growth form	Native forb	44
	Exotic forb	31
	Native grass	24
	Exotic grass	10
Origin × grass type	Native C3 grass	16
	Native C4 grass	8
	Exotic C3 grass	9
Origin × lifespan × growth form	Native annual forb	7
	Native perennial forb	37
	Exotic annual forb	26
	Exotic perennial forb	5
	Exotic annual grass	9
	Exotic perennial grass	1
Origin × growth form × N-fixing	Native nonlegume forb	40
	Native legume forb	4
	Exotic nonlegume forb	24
	Exotic legume forb	7

Note: Taxa were assigned to groups hierarchically (e.g., we analyzed all native species, and further divided them into native forbs and native grasses, etc.), to examine broad responses while investigating functional traits driving broader patterns.

[a]The C4 grass group is encapsulated by the Native C4 grass group as all C4 grasses in our study system are native.

in academic science, we also classified taxa into five categories used by the local government to monitor grassland quality (Australian Government, 2017; Rehwinkel, 2015): (1) Indicator A, native taxa that are uncommon or rare, sensitive to disturbance and indicate high-value native grassland; (2) Indicator B, native taxa that indicate high-value native grassland but are more common than A and tolerate some disturbance; (3) Indicator X/Y, high-impact exotic taxa, often declared noxious weeds

and difficult to manage; (4) Indicator Z, low-impact, mostly annual weeds; and (5) Common/Increaser species, native and exotic taxa that tolerate or respond positively to disturbance. Taxa were assigned to 27 functional groups based on these categories in a hierarchical way and these groups were used in the analysis. For example, we analyzed the richness and diversity of all taxa, native taxa and exotic taxa separately, while native and exotic grasses were further divided into C3 and C4 categories (Table 1). This allowed us to analyze broad functional responses while also examining which functional traits drove broader patterns.

For each functional group in each quadrat, we calculated species richness (number of species) and, to account for variation in abundance and evenness among species, the Inverse Simpson's Diversity Index. We used this index as a measure of biodiversity because it does not inflate the zeros in the data; richness values of one had diversity values of one, unlike other diversity metrics where richness values of one equal zero.

Analysis

To analyze the influence of the spray method (a three-level factor) and *H. perforatum* density (a two-level factor) on the proportion of the water-sensitive card affected by herbicide (Question 1), we fitted a beta regression model with a logit link function using the *mgcv* package (Wood, 2011) in R (R Core Team, 2022). We included the main effects of the two terms and their interaction to determine whether the influence of the spray method differed depending on the density of *H. perforatum*. This model included the fixed effects only. To examine differences between treatment levels within the fitted variables (e.g., spot spray vs. fine boom), we calculated the least-squares means contrasts and 95% CI on the link scale using custom scripts (Smith et al., 2023). We inferred important differences when the confidence interval did not include zero.

To analyze the influence of the spray method on the occurrence, richness and diversity of plant functional groups, and on the presence and abundance of individual native taxa we used generalized linear mixed models (GLMM) in R. Each analysis used a different GLMM family, appropriate for the data type, but we used the same model structure and approach for all data types. Our primary hypothesis was that there would be a two-way interaction between year and spray method, reflecting the before-after-control-impact nature of the study design. That is, the effect of the spray method should not be detectable in 2017 before the treatments were applied; any effects should only become apparent in 2018 and 2019. However, we also wanted to determine whether these effects were generalizable across space, thus we first fitted a three-way interaction between year (a linear numeric variable corresponding to the three survey years), spray method (a factor with three levels: control, spot spray and boom spray) and reserve (a factor with two levels: Jerrabomberra and Mulanggari). A detectable three-way interaction would indicate differential effects of herbicide in the two reserves which vary in initial plant species composition. We used a *p*-value to determine the importance of the three-way interaction, and removed the three-way term if $p \leq 0.05$. In cases where the three-way term was removed, our final model was year × spray method + reserve. We did not simplify the model further as this corresponded to our primary hypothesis. Support for this model (two-way interaction $p < 0.05$) would indicate that the effects of the spray method were consistent across reserves, while also accounting for overall differences in the response variable between reserves (a main effect of reserve). We calculated least-squares mean contrasts among treatment levels within years, within reserves, as described for the spray drift analysis.

Our analysis goal was to examine variation in species richness and diversity of the 27 functional groups. When data are limited, however, such as with rare or spatially patchy functional groups, important effects can be missed with models for count or continuous numeric data as they are typically data hungry. Thus, we began by modeling the probability of occurrence (presence/absence) for 10 of the 27 functional groups with more than >20% zeros (absences) across the 144 observations in the data (Appendix S1: Table S1). For these groups, we modeled probability of occurrence using binomial GLMMs with a logit link in the *lme4* package (Bates et al., 2015). This allowed us to determine whether spraying affected the probability of detecting any plant in those rare or patchy groups.

For all functional groups, we then modeled species richness (count data) with a negative binomial distribution and the Inverse Simpson's Diversity Index with a gamma distribution (appropriate for positive, continuous numeric data with heterogeneity, i.e., over-dispersion). Both of these model types were fitted in the *glmmADMB* package (Fournier et al., 2012; Skaug et al., 2016) in R. We excluded exotic perennial grass and sedge/rush from these analyses because they had fewer total observations than the number of quadrats in the study ($n = 5$ and $n = 27$, respectively; Table 1).

To examine the effects of the spray method on the occurrence and abundance of individual native forb species (the growth form most likely affected by broadleaf herbicide), we focused on the five species used to define

the study plots and three other species commonly found in our study region (described above and in Appendix S1: Table S2). For all eight species, we first modeled probability of occurrence using binomial GLMMs with a logit link function. We then modeled abundance of individual species, conditional on presence (i.e., the positive values in the data) using a truncated negative binomial model in *glmmADMB*. We were unable to model abundance for species with few or strongly clustered data (>50% zeros), leaving four species in our abundance analysis: *Chrysocephalum apiculatum*, *Eryngium ovinum*, *Lomandra filiformis coriacea* and *Tricoryne elatior* (Appendix S1: Table S2).

RESULTS

Overview

We recorded 119 species during the study, of which 78 (66%) were native (Table 1). In general, herbicide application led to a decline in the occurrence and abundance of native forbs, while causing an increase in the richness of exotic plant functional groups (those not normally targeted with herbicide; Appendix S1: Tables S1 and S2). These effects were most pronounced for spot spraying. Effects of herbicide were evident only for forbs, except for an increase in exotic species richness overall, which was likely to have been driven by the response of exotic forbs. Grasses were not affected by herbicide nor was the sedge/rush group, although the latter had too few data to draw reliable conclusions. None of the "indicator" groups responded to herbicide (Appendix S1: Table S1). Where significant main effects of reserve were found, all functional groups, except native C3 grasses, were more diverse at Mulanggari than Jerrabomberra, across all years (Appendix S1: Table S3).

Herbicide application method (Question 1)

The model examining the proportion of water-sensitive cards affected by spraying indicated an effect of the spray method ($p < 0.001$) but no effect of *H. perforatum* density ($p = 0.965$) or the interaction between spray method and *H. perforatum* density ($p = 0.668$). Spot spraying was estimated to cause near complete covering of the water-sensitive cards (91%–99%) which was significantly higher than both boom spray methods (Figure 1). The two boom spraying methods only partially covered the cards (5%–31%) and were not significantly different from one another (Figure 1).

Effect of herbicide on resident communities (Question 2)

We found the effects of fluroxypyr on the probability of occurrence of two of the 10 functional groups analyzed with binomial models (Appendix S1: Table S1). For native leguminous forbs, there was a significant three-way interaction ($p = 0.031$), indicating that the interactive effect of the spray treatment across years differed between the two reserves (Figure 2a,b). This model showed a significant decline in the occurrence of native leguminous forbs with spot spraying at the *Themeda* grassland, Mulanggari (Figure 2a). There was also a clear decline in the occurrence of native leguminous forbs with both spot and boom spraying at the *Austrostipa/Rytidosperma* grassland, Jerrabomberra. However, this functional group was present on all control quadrats in these years and contrasts on the link scale were not reliably estimated because the fitted values were approaching the boundary of the model (Figure 2b). For exotic leguminous forbs there was a consistent interaction between year and spray method across reserves (two-way interaction, $p = 0.003$). Spot spraying led to a higher probability of the occurrence of exotic leguminous forbs at both reserves, while boom spraying had no effect on this functional group (Figure 2c,d).

For species richness, we found significant interactions for three of the 25 functional groups analyzed (Appendix S1: Table S1). There was a significant two-way
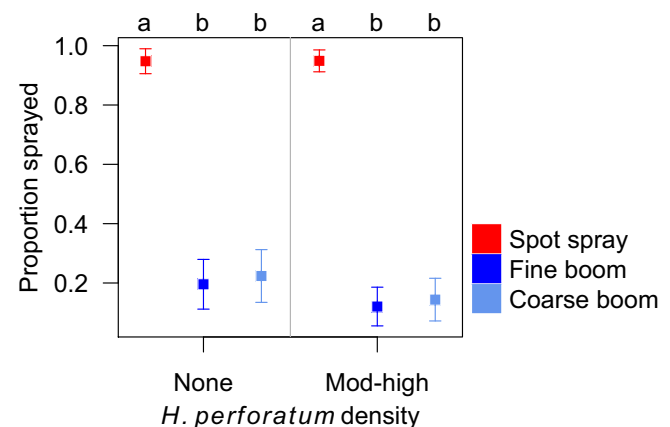

FIGURE 1 The effect of three different spraying methods and in situ *Hypericum perforatum* density (none or moderate to high) on the proportion of water-sensitive cards affected by spraying (model estimates and 95% CI, on the response scale). Model *p*-values were: spray method <0.001; *H. perforatum* density = 0.965, method × density = 0.668. Shared letters (a, b) indicate no difference. *Hypericum perforatum* is the target species regularly sprayed in our study system. An example of the water-sensitive card method is shown in Appendix S1: Figure S1.
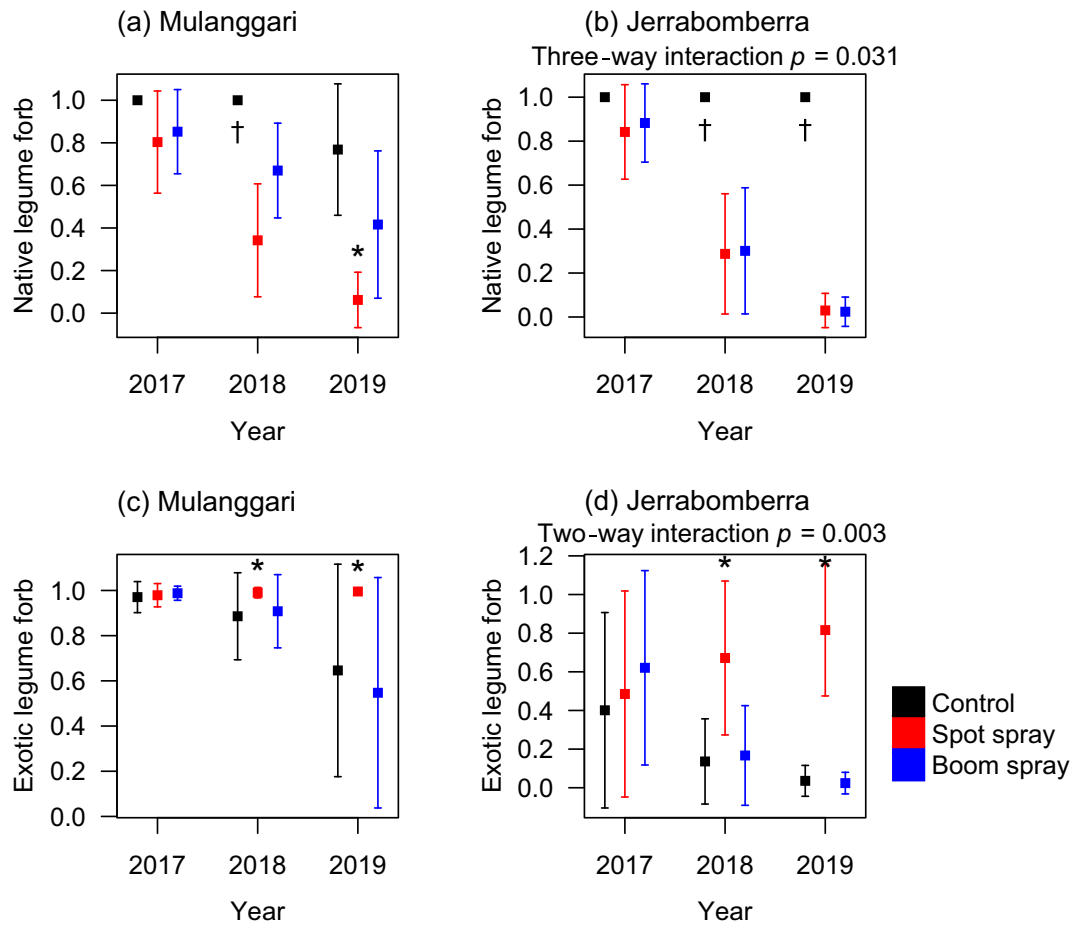
FIGURE 2 The effect of year and spray method on the probability of occurrence of plant species modeled as functional groups. (a, b) For native leguminous forbs the interactive effect of the spray treatment across years differed between the two reserves (three-way interaction) while for (c, d) exotic leguminous forbs there was a consistent interaction between year and spray method across reserves (two-way interaction). Mulanggari is a *Themeda* (C4) grassland and Jerrabomberra is *Austrostipa/Rytidosperma* (C3) grassland. Estimates and 95% CI are shown on the response scale (probability). The asterisks (*) indicate treatment levels that were different from the control, on the link (logit) scale, within years, within reserves. †Native leguminous forbs were present on all control quadrats in these years, and a decline in occurrence was observed in both the spot spray and boom spray quadrats. However, contrasts on the link scale were not reliably estimated between the control and boom treatments because the fitted values were approaching the boundary of the model.

interaction between year and spray method for all exotic species ($p < 0.001$; Figure 3a,b) and exotic forbs ($p = 0.002$; Figure 3c,d). Spot spraying caused an increase in the richness of these functional groups by the end of the experiment in 2019. The positive effect of spot spraying was also evident in 2018 for all exotic species (Figure 3a,b). For exotic annual forbs, there was a significant three-way interaction between year, spray method and reserve ($p = 0.039$). Spot spraying led to a significant increase in the richness of this group at Jerrabomberra but not Mulanggari (Figure 3e,f). Overall, the species richness results suggested the effect of spraying on all exotic species was likely to have been driven by the responses of forbs, because no other exotic functional groups at a finer level

responded to the herbicide (Appendix S1: Table S1). There were also the main effects of year on all of these three groups, with richness declining between 2017 and 2019 (Figure 3).

For the Inverse Simpson's Index, we found significant interactions for three of the 25 functional groups analyzed (Appendix S1: Table S1). However, interactions for two groups (Indicator B and exotic leguminous forbs) were driven by differences between reserves, and did not relate to differences among herbicide treatment levels within years within reserves (Appendix S1: Figure S2a,b,e,f). For exotic annual forbs, the interactive effect of the spray method across years differed between the two reserves (three-way interaction, $p = 0.014$). This model
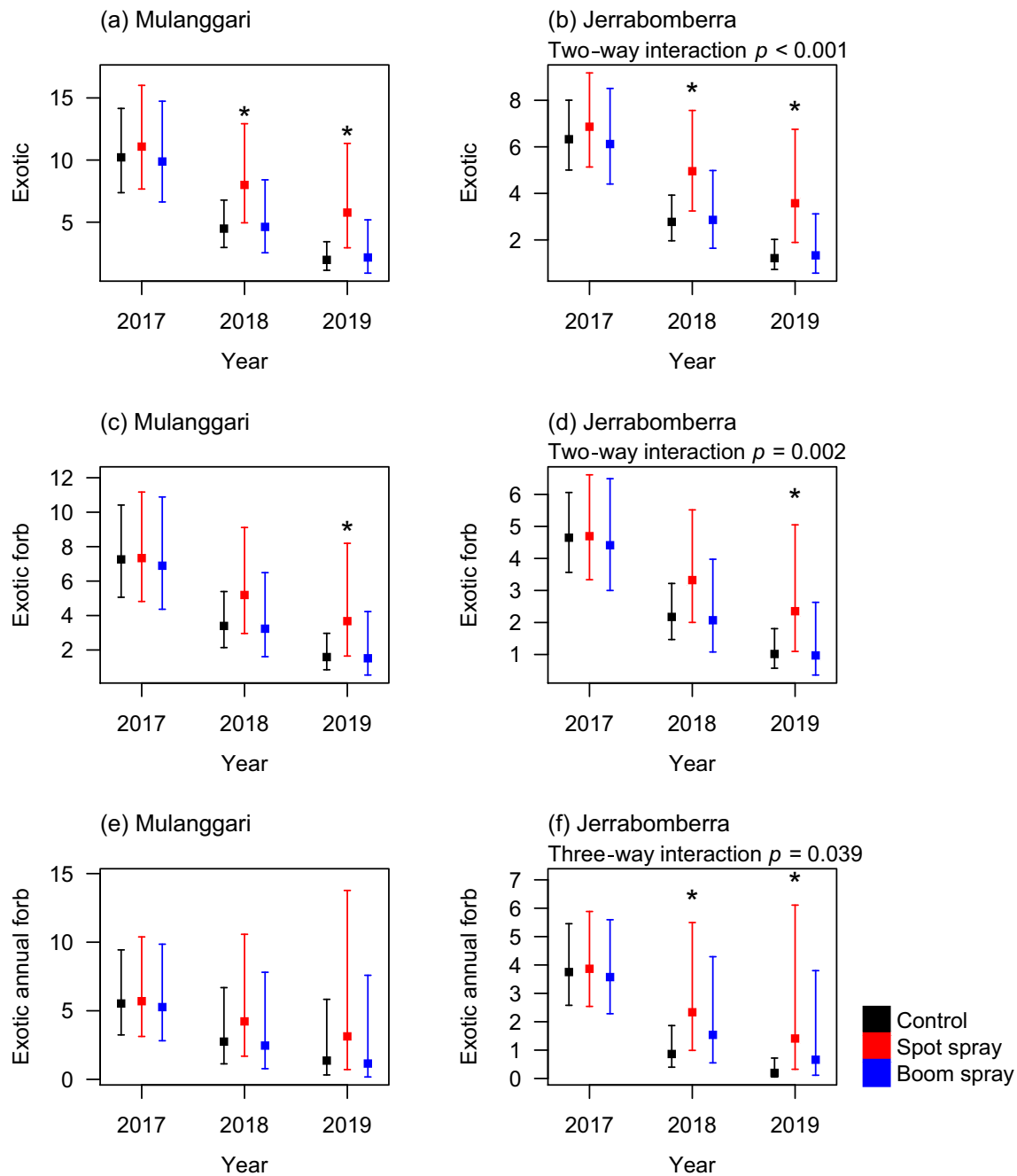
FIGURE 3 The effect and of year and spray method on species richness of plant functional groups. For (a, b) all exotic plants and (c, d) exotic forbs there was a consistent interaction between year and spray method across the two reserves (two-way interaction). (e, f) For exotic annual forbs the interactive effect of the spray treatment across years differed between the two reserves (three-way interaction). Mulanggari is a *Themeda* (C4) grassland and Jerrabomberra is *Austrostipa/Rytidosperma* (C3) grassland. Estimates and 95% CI are shown on the response scale (richness). The asterisks (*) indicate treatment levels that were different from the control, on the link (log) scale, within years, within reserves.

showed that spot and boom spraying caused an increase in exotic annual forb diversity at Jerrabomberra but not Mulanggari (Appendix S1: Figure S2c,d). Thus, accounting for relative abundance revealed an effect of boom spraying that was not evident in the species richness analysis, where only spot spraying increased exotic annual forb richness (Figure 3e,f).

Effect of herbicide on individual forbs (Question 3)

We found an effect of herbicide on the probability of occurrence of one of the eight species analyzed (*D. varians*) and on the abundance of two of the four species analyzed (*C. apiculatum* and *E. ovinum*; Figure 4;
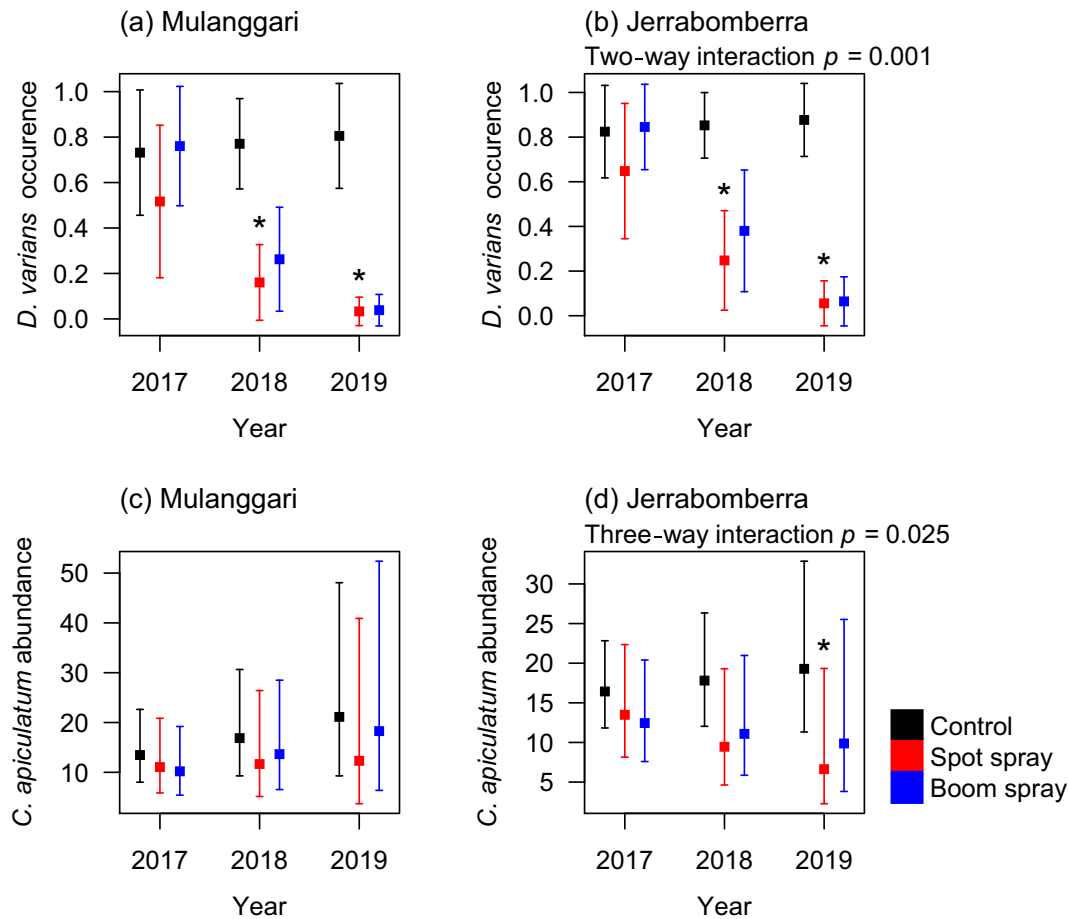
FIGURE 4 The effect of year and spray method on individual native plant species. (a, b) The probability of occurrence of *Desmodium varians* was affected by a consistent interaction between year and spray method across the two reserves (two-way interaction). For the abundance of (c, d) *Chrysocephalum apiculatum*, the interactive effect of the spray treatment across years differed between the two reserves (three-way interaction). Mulanggari is *Themeda* (C4) grassland and Jerrabomberra is *Austrostipa/Rytidosperma* (C3) grassland. Estimates and 95% CI are shown on the response scale (richness). The asterisks (*) indicate treatment levels that were different from the control, on the link scale (a, b logit; c, d log), within years, within reserves. Contrasts between the control and boom treatments (a, b) were not reliably estimated because the fitted values were approaching the boundary of the model.

Appendix S1: Table S2). For *E. ovinum* however, there was an inexplicable treatment effect evident before herbicide was applied in 2017, thus we were unable to draw reliable conclusions about this species (Appendix S1: Figure S3). Spot spraying caused a consistent decline in the occurrence of *D. varians* at both locations (two-way interaction, $p = 0.001$). This decline was also evident for boom spraying although contrasts on the link scale were not reliably estimated because the fitted values were approaching the boundary of the model (Figure 4a,b). For *C. apiculatum*, the interactive effect of the spray method across years differed between the two reserves (three-way interaction, $p = 0.025$). Spot spraying caused a decline in *C. apiculatum* abundance at Jerrabomberra but not Mulanggari (Figure 4c,d).

DISCUSSION

In threatened temperate grasslands managed primarily for conservation, we found that the application of herbicide to control the invasive *H. perforatum* led to a short-term (3-year) decline in the occurrence and abundance of native forb functional groups and individual species. At the same time, herbicide led to an increase in the occurrence and richness of exotic forbs, a functional group not usually targeted by herbicide application. Spot spraying had more negative impacts on native species and more positive impacts on exotic species than boom spraying, although some effects were evident for both methods. Our direct measurements of spray coverage showed that spot spraying almost completely covered the ground surface, and suggest that native forbs would be heavily

impacted by this method. To assist land managers in deciding when and how to control the invasive plant *Hypericum perforatum*, we constructed a decision tree that combined the outcomes of this study with previously published literature and our on-ground field experience in managing grasslands (Appendix S1: Section S1). The decision tree shows that boom spraying should be used when *H. perforatum* density is moderate to high and spot spraying (often at reduced pressure) should only be used when *H. perforatum* density is very low (Appendix S1: Figure S4). Given regional variation in *H. perforatum* density, spatial scale of invasion, soil depth, and conservation values, the decision tree allows managers to evaluate the costs and benefits of chemical control and indicates situations where alternative or modified methods could be used (Appendix S1: Figure S4). Because this was a short-term study, research is needed to examine whether native species can recolonize in the longer term following herbicide application.

Fluroxypyr is a broadleaf herbicide that, in our study system, is assumed to have minimal effects on nontarget species because it does not negatively impact grasses and annual forbs can regenerate in the following growing season (Campbell & Nicol, 1997). However few, if any, studies have quantified its impact across the whole grassland community. Our results are interesting first because they disprove these assumptions—fluroxypyr negatively impacts native forb species—and second because they suggest that fluroxypyr can increase the richness and diversity of exotic forbs. Native species in our system might therefore be impacted directly by the chemical but also indirectly by competition with exotics. Our study was not designed to differentiate between these potential mechanisms, but there is evidence for both chemical (Carpenter et al., 2020) and competitive (Byun et al., 2013) processes in the literature.

Competitive displacement of native species by exotics has been documented in grassland communities elsewhere (Thiele et al., 2010) and how this plays out depends on the traits of the invader as they relate to the resident community (Britton et al., 2021). In our study, exotic forbs responded positively to herbicide spot spraying and, at one location, this appeared to be driven specifically by exotic annuals. Other studies have shown annual forbs to be strong early invaders (e.g., Byun et al., 2013; Galland et al., 2019), while perennial species are likely to have a stronger invasibility in the longer term (e.g., > 5–10 years, Catford et al., 2019). Our study covered only 3 years, so different functional groups (native or exotic) might respond differently to herbicide over longer timescales and continued monitoring is needed to understand these effects.

A potential reason for the increase in the occurrence of exotic leguminous forbs specifically, is that they had died back or slowed their growth at the time of summer spraying (all exotic legumes in our study were annual, cool-season *Trifolium* species). Their positive response to herbicide could have been facilitated by reduced competition with native legumes that were directly affected by fluroxypyr during their active growing period. This would require the exotic species to recruit from a soil-stored seed bank or through dispersal from surrounding areas, either of which is possible given their high fecundity and small seeds (Boswell et al., 2003). Herbicides can negatively impact seed viability in exotic and native plants (Carpenter et al., 2020; Steadman et al., 2006) but it does not appear to have affected exotic species in our study (possibly because the seed of many exotics had matured before spraying). In addition to the positive influence on exotic leguminous forbs, we also found that spot spraying increased the richness of exotic species generally, including grasses. It is possible that these results are linked as legumes could have enhanced the ability for a greater diversity of exotic species to establish by increasing N availability (Frankow-Lindberg, 2012). The increase in exotic forb species richness with spot spraying occurred despite the observed declines in richness over time. These declines probably reflect the prolonged drought between 2017 and 2019, coinciding with the years of the current study, when record low rainfall occurred (Australian Government Bureau of Meteorology).

A final possibility relating to competitive processes is that, compared with native species, there were more exotic annual (26 vs. 7) and exotic leguminous forb (7 vs. 4) species in these grasslands (Table 1). Thus, even if both groups were similarly impacted, a greater proportion of exotic species groups could have avoided the strong effects of herbicide by chance because of their higher occurrence. Regardless of the underlying mechanism, the competitive effects of invading species might increase as the climate warms by increasing the impact on the individual fitness of resident species (Britton et al., 2021). Any practice that gives exotic species a further competitive edge, such as herbicide appeared to do in this study, should therefore be avoided.

Fluroxypyr mimics the regulation of auxin, a plant hormone involved in growth and development (Woodward & Bartel, 2005) and mutations at an auxin signaling gene are involved in the development of fluroxypyr resistance (LeClere et al., 2018). By late 2021, herbicide resistance had been documented in 266 species globally, 82 of which were resistant to auxin mimics and four of which were forbs specifically resistant to fluroxypyr (Heap, 2021). One of these is *Gallium aparine* and our data included two exotic *Gallium* taxa (*G. divaricatum* [n individuals = 244] and an

unidentified *Gallium* species [$n = 3$]). We are not suggesting that our results reflect evolved fluroxypyr resistance, but highlight that some species, especially invasive forbs, are not susceptible to the chemical pathway on which fluroxypyr acts (Hill et al., 1996). Introduced invasive species often have high levels of genetic diversity, which promotes the rapid spread of favorable alleles and thus adaptation to a wide range of environmental conditions (Smith et al., 2020; van Boheemen et al., 2019; Wilson et al., 2009). Many exotic forbs in our study system are invasive in Australia (e.g., *Hypochaeris radicata*, *Plantago lanceolata*, *Taraxacum* spp., and *Trifolium* spp., McDougall et al., 2005; Smith et al., 2020). Thus, if the documented increases in exotic forbs following fluroxypyr application reflected tolerance to the chemical (founded on high levels of genetic and phenotypic variability), exotic forbs could exploit the space and nutrient availability left following declines in native forbs. More research is needed to quantify this risk.

Land managers often assume that spot spraying is the most ecologically sensitive method of herbicide application due to the operator's ability to specifically target weeds and presumably reduce nontarget impacts (GeFellers et al., 2020; Hunt, 2017). Our results showed the opposite, that spot spraying covered more surface area than boom spraying. However, we found no effect of *H. perforatum* density on surface coverage, all three treatments covered approximately the same surface area across two contrasting levels of *H. perforatum* density. Despite this, the scale of the impact of fluroxypyr will depend on the scale and magnitude of *H. perforatum* infestation simply because increased amounts of chemical will be applied when there is a greater density of the target weed. In our experiment, we applied spot spraying consistently, at the same rate across both density levels. In reality however, herbicide application rate is not set with the spot spray apparatus and operators can easily overapply herbicide. When target weed density is high to moderate, this will translate to a high chemical load as the operator will spray more plants. This was clearly indicated on our water-sensitive cards and reflected in the plant community data. Reducing the pressure of the spot sprayer (e.g., from 15 to 5 kPA) could substantially minimize negative effects on native species, while still applying enough herbicide to control the target invader (Appendix S1: Figure S4).

To assist land managers in deciding when and how to control the invasive plant *Hypericum perforatum*, we constructed a decision tree that combined the outcomes of this study with previously published literature and our field experience in managing grasslands (Appendix S1: Section S1, Figure S4). The decision tree is structured into four branches: Branch (1) *H. perforatum* density; Branch (2) spatial scale of infestation (e.g., landscape scale vs. isolated); Branch (3) conservation (floristic) value of the site; and Branch (4) soil depth. The physical and ecological conditions of sites can be assessed at each branch to determine a final recommendation for the control method (provided in the fifth column of the decision tree). Given the many competing priorities handled by land management agencies, the decision tree allows for "no treatment" in grasslands with low conservation value, where *H. perforatum* density is low or unlikely to have a significant impact, such as in shallow soils (Appendix S1: Figure S4).

In our study system, the use of herbicide to control *H. perforatum* is based largely on its legal status as a declared pest and assumptions about its impact on biodiversity (Campbell & Nicol, 1997). However, we still have little knowledge about the extent to which *H. perforatum* impacts native biodiversity, an essential topic for future research. It is possible that chemical control is doing more harm to these threatened grasslands than the weed itself and this must be quantified. Our data suggest that, when *H. perforatum* density is moderate to high, methods of weed control other than herbicide should be investigated and implemented. In the same study system, we have found annual mowing (Smith et al., 2018) and prescribed fire (McDougall et al., 2016) have led to positive biodiversity outcomes for plant communities and threatened animal species. Careful application of prescribed fire might be especially important in these grasslands, not so much for its ability to reduce *H. perforatum* density (which can rapidly resprout following a fire, Briese, 1996) but for its ability to promote the establishment and growth of native grasses, which confer ecosystem-level resistance to invasion by exotic species (Morgan & Salmon, 2020; Prober & Lunt, 2009; Prober & Thiele, 2005). Thus, fire, combined with revegetation of foundation grass species where propagule pressure is low, might be an optimal way to restore native grasslands while avoiding secondary invasion (Pearson et al., 2016). Although this might be more costly in the short term, focusing on ultimate restoration goals rather than short-term solutions can be more cost effective in the long run (Prior et al., 2018) while increasing the likelihood of effective biodiversity conservation. Given the declines in native forbs with herbicide that we documented here, this longer term strategy that minimizes chemical inputs seems warranted.

AUTHOR CONTRIBUTIONS

Richard N. C. Milner, Jack French, and Annabel L. Smith designed the study; Jack French undertook the treatments; Tobias Hayashi and Richard N. C. Milner collected the data; Annabel L. Smith and Richard N. C. Milner designed the analytical approach; Annabel L. Smith and Raagini M. Kanjithanda analyzed the data; Annabel L. Smith wrote

the paper with input from Richard N. C. Milner, Raagini M. Kanjithanda, Tobias Hayashi, and Jack French.

CONFLICT OF INTEREST STATEMENT
The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT
Data and code (Smith et al., 2023) are available in Zenodo at https://doi.org/10.5281/zenodo.7847738.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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