Investigations into suitability of *Trifolium occidentale* as a host plant for two common pasture pests

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Abstract Western clover (*Trifolium occidentale*) is a diploid perennial clover that is reported to be one of the progenitors of white clover (*Trifolium repens*). The ability to produce hybrids between *T. repens* and *T. occidentale* provides an opportunity to introduce factors to improve white clover tolerance to common stress factors. A series of assays was undertaken to compare the feeding and performance of two contrasting pests on two *T. occidentale* lines and two *T. repens* cultivars. Clover root weevil (*Sitona lepidus*) adults showed a preference for *T. repens*, but this diminished if weevils had been previously exposed to *T. occidentale*. Weevil longevity, feeding levels and oviposition were comparable over 32 days, indicating *T. occidentale* is a host plant for adult *S. lepidus*. Clover flea (*Sminthurus viridis*) showed a strong preference for *T. repens* over *T. occidentale* in a choice test and higher feeding levels on *T. repens* in the no-choice test.

Keywords choice test, no-choice test, *Sminthurus viridis*, *Sitona lepidus*.

INTRODUCTION

Western clover (*Trifolium occidentale* Coombe) is a diploid stoloniferous perennial clover originating from coastal habitats, especially rocky outcrops, on the Gulf Stream coasts of Portugal, Spain, France, Ireland and the United Kingdom. The species so closely resembles white clover (*Trifolium repens* L.) that it was not recognised as a separate species until 1960 (Coombe 1961). It is of high interest to white clover breeders because the white clover allotetraploid genome is derived from *T. occidentale* and another diploid ancestor, *T. pallescens* Schreb. (Williams et al. 2012). It is a useful model plant for determining gene function in white clover as it has a small genome, forms hybrids with *T. repens*, has both self-fertile and self-incompatible breeding lines, a short generation time, and forms the same symbiotic relationship with *Rhizobium* spp.

bacteria (Williams et al. 2009). In addition, it is considered a useful genetic resource to improve the resistance of white clover cultivars to common stressors such as diseases and drought (Pederson & McLaughlin 1989; Williams et al. 2009).

The arrival of clover root weevil (*Sitona lepidus* Gyllenhal) in New Zealand highlighted the vulnerability of modern clover cultivars to pests (Crush 2005). As the native distribution of *S. lepidus*, a specialist *Trifolium* spp. herbivore, overlaps that of *T. occidentale*, the clover may also have useful heritable mechanisms to protect against attack. While it has been shown *T. occidentale* can host the nematodes *Meloidogyne trifoliophila*, *M. hapla*, *M. incognita* and *M. javanica* (Mercer & Miller 1997), there is no published information on whether the clover is a suitable host for *S. lepidus* or other common clover pests.

The simplest and most common first step in assessing if a plant is acceptable as a host plant is a feeding assay where level of feeding on the test plant is compared with that on a known host plant. *Sitona lepidus* adults make a distinctive U-shaped notch on the leaflet edge at each feeding episode, the size of which varies with adult size. Therefore *S. lepidus* feeding activity is usually assessed by counting notches (Murray & Clements 1994; Murray 1996a; Murray & Willoughby 1998; Gerard & Crush 2003). In addition, when in a reproductive state, *S. lepidus* females lay eggs continuously if provided quality clover foliage and these can be readily collected from the cage base. Thus egg counts obtained during no-choice feeding assays can provide additional information on *S. lepidus* host plant suitability (Murray 1996b; Gerard & Crush 2003). In contrast, clover flea (*Sminthurus viridis* L.) feeds on the mesophyll (Maclagan 1932) leaving one cuticle layer of the leaf intact. The combination of relatively poor feeding scar definition and the abundance and size variation in scars makes direct counts or leaf area scans difficult. Therefore, most authors have visually scored the severity of damage on control and test plants (Cooper 1994; Bishop & Barchia 2003).

The objective of this study was to compare levels of foliar feeding on *T. repens* and *T. occidentale* by adult *S. lepidus* and *S. viridis*, a generalist herbivore that is a sporadic and at times a serious pest of white clover.

MATERIALS AND METHODS

The experiments were run at the Ruakura Research Centre, Hamilton, in summer 2013/14. Seeds of two *T. repens* cultivars (Grasslands Kopu II and the inbred Crau S9 2A) and two *T. occidentale* populations (48 and AZ4270) were obtained from the Margot Forde Forage Germplasm Centre at Palmerston North. Kopu II was selected as an industry standard while the inbred clover was reported to be less resilient to common stresses than outcrossed clovers. Over 60 plants of each clover (hereafter referred to as plant lines) were grown in commercial potting mix, in individually-labelled pots in a glasshouse.

Within each assay (described below), a leaf from a different plant was used in each replicate. Care was taken to select mature leaves of approximately equal size, which meant picking the smaller *T. repens* leaves and the largest *T. occidentale* leaves. Where this was not possible, additional *T. occidentale* leaves were added to give a visually approximately equal leaf area.

The invertebrates were collected from clover patches in nearby ryegrass/*T. repens* pastures using a modified blower-vac and were hand-sorted in the laboratory.

Sitona lepidus assays

Choice tests were carried out in numbered Petri dishes lined with damp filter paper that had been marked into four labelled quarters with pencil. Clover leaves of similar size taken from each plant line were placed in the appropriate quadrat and the plant identification recorded. One *S. lepidus* adult from the field collection was placed in each Petri dish. There were 20 replicates and the assay was held in a controlled environment room at 20°C and 16 h photoperiod. The number of feeding notches on each leaf was assessed at 24 h.

A no-choice assay was run in vented 100 ml specimen vials with one clover leaflet and one weighed female weevil per vial. The vials were used rather than Petri dishes as vials simplified maintenance and assessments, and reduced the chance of handling injuries to weevils during these processes. The weevils had been caged individually beforehand and only those that had laid eggs were used. There were 14 replicates for each plant line and the assay was held in a controlled environment room at 20°C and 16 h photoperiod. After 24 h, notches and the number of eggs laid were recorded. The vial was cleaned and the old leaf was replaced with a fresh leaf from the same plant. The assay continued over the next 32 days with assessments as time permitted (17 in total). When more than 48 h had passed since the previous assessment, only the number of eggs was recorded.

A second choice test was carried out using weevils from the no-choice assay above to ascertain if feeding preference was modified by

previous 32 day experience. Petri dishes were lined with damp filter paper that had been marked into two labelled sectors with pencil. Clover leaves of similar leaf area from *T. repens* cv. Kopu II and *T. occidentale* 48 were placed in the appropriate sector and a single *S. lepidus* that had been feeding solely on either *T. repens* Kopu II or *T. occidentale* AZ 4270 for the previous month was added to each dish. There were 12 replicates of weevils from each treatment and the assay was held in the same conditions and assessed as described in the above choice assay.

Sminthurus viridis assays

Choice and no-choice assays were carried out in test apparatus described by Eden et al. (2005). *Sminthurus viridis*, freshly collected from the field, were caged in vented 100 ml specimen vials and the petioles of the test clover leaves were threaded through holes into a water source below. Around 1 mm of sieved soil was put in the base of the cages; this improves *S. viridis* longevity by absorbing and buffering condensation.

In the no-choice assay, there were 10 replicates for each of the four plant lines, each consisting of a single leaf and a single *S. viridis*. The choice assay was set up in an identical manner, except each vial contained a *T. repens* Kopu II leaf (identified by a mark next to the hole for the petiole) and a leaf from one of the other three clovers.

The assays were held in a controlled environment room at 15°C and 16 h photoperiod and assessed at 24 h. *Sminthurus viridis* survival was assessed and feeding damage on each leaflet of the trifoliate leaf was scored from 0 (no damage) to 4 (over half leaf area damaged) and added to obtain an overall score for each leaf. No-choice replicates with dead *S. viridis* were rerun using a new *S. viridis* to investigate if mortality was related to the test clover.

Statistical analyses

The *S. viridis* data and *S. lepidus* choice data were analysed by ANOVA, and means in the choice tests compared using a paired t-test. The *S. lepidus* no-choice data were square-root transformed and ANOVA was conducted with

weevil weight as a covariate. The relationship of weevil weight, feeding notches and eggs laid was investigated using regression analysis.

RESULTS

Sitona lepidus assays

When given a choice, *S. lepidus* adults showed a significant preference for *T. repens* over *T. occidentale* (Table 1), but differences between plant lines within the same species were not significant. In the second choice assay, previous feeding treatment influenced the acceptability of *T. occidentale* (Figure 1). Thus, those weevils pre-fed *T. repens* showed a significant preference for *T. repens* ($P < 0.001$), whereas the preference between *T. repens* and *T. occidentale* was statistically insignificant for those pre-fed on *T. occidentale* for 32 days. Weevils that had previously fed on *T. occidentale* tended to feed more than those that had previously fed on *T. repens* but this was not statistically significant ($P = 0.085$).

Figure 1 Effect of previous clover diet on feeding notches produced by *S. lepidus* adults after 24 h when given a choice of *T. repens* and *T. occidentale*. Error bars denote SE for total notches.

Only two weevils died, one from each plant species, during the no-choice assay. Within plant species, a similar level of notching was recorded. While it does appear fewer eggs were laid by adults held on *T. occidentale* AZ4270 compared to the other clovers (Figure 2), the differences were not statistically significant when adult weight was used as a covariate. The total numbers of eggs

Table 1 Mean number of notches on leaves after 24 h when *S. lepidus* adults had the choice of four clovers.

Species	Plant line	Notches (plant species)	Notches (plant line)
<i>T. repens</i>	Kopu II	1.35	1.15
	Crau S9 2A		1.55
<i>T. occidentale</i>	AZ4270	0.375	0.30
	48		0.45
LSD (P=0.05)		0.52	0.71

laid per adult was correlated with adult weight ($P<0.001$; $y = 15x - 9.5$, $R^2=0.22$) and while weevil weight was relatively well distributed across the clover treatments ($P=0.21$), there was large variability in eggs laid, especially towards the end of the experiment. The number of eggs laid per adult declined after a peak in week 2, with over 35% of weevils having ceased oviposition by the final assessment.

Figure 2 Pattern of oviposition by *S. lepidus* adults when individually maintained on four different clovers over 5 weeks. Means adjusted for covariation in adult weights. Error bars denote SED.

Sminthurus viridis assays

In the no-choice assay, damage by *S. viridis* was significantly greater on *T. repens* than on *T. occidentale* ($P<0.001$), and there was no difference between CrauS9 2A and *T. occidentale* or plant lines within species (Table 2). There was no effect on clover flea survival. When *S. viridis* were given a choice between *T. repens* cv. Kopu II and one of the other three clovers, Kopu II was preferred, though the difference was only significant for *T. occidentale* AZ4270 ($P<0.01$) (Figure 3).

Figure 3 Comparison of feeding severity when *S. viridis* was given the choice between *T. repens* cv. Kopu II and a test clover. Error bars denote SE.**Table 2** Mean *S. viridis* damage scores on leaves from four clovers after 24 h in a no-choice assay.

Species	Plant line	Score (plant species)	Score (plant line)
<i>T. repens</i>	Kopu II	3.53	4.03
	Crau S9 2A		3.03
<i>T. occidentale</i>	AZ4270	1.86	1.97
	48		1.74
LSD (P=0.05)		0.74	1.40

DISCUSSION

Previous studies have shown that *S. lepidus* adults feed almost exclusively on *Trifolium* spp. and have preferences within the genus, with *T. repens* being highly preferred and *T. dubium* Sibth. rejected (Murray & Clements 1994; Hardwick 1998). There are also within-species differences. For instance, reduced feeding, adult longevity and oviposition have been associated with red clover *T. pratense* L. breeding lines containing high phyto-oestrogen levels (Gerard et al. 2005; Murray et al. 2007). In contrast, *S. viridis* has a wide host range (Holdaway 1927). However, it is known mainly as a pest of Fabaceae, in particular *T. repens* and lucerne (*Medicago sativa* L.). *Sminthurus viridis* has also shown feeding preferences between cultivars (Cooper 1994).

Assays in the present study show that *T. occidentale* is a suitable host plant for adult *S. lepidus* and testing is currently underway to confirm it supports larval development. While adults that had never previously fed on *T. occidentale* preferred *T. repens*, there is evidence that they become habituated to *T. occidentale* following exposure. In addition, the pattern of feeding, oviposition and survival on *T. occidentale* appeared similar to that on *T. repens* over the 32-day assessment. The termination of oviposition by adults in this experiment was not associated with feed quality, rather it is thought to be associated with depletion of sperm in these long-lived weevils that would normally mate more frequently than 30 days. *Sitona lepidus* females store sperm in their spermathecae and cessation of oviposition by females with empty spermathecae has been reported previously (Gerard et al. 2005).

Sminthurus viridis also showed a preference for *T. repens* over *T. occidentale*, but as the assays were limited to 24 h, it is not known if prolonged exposure influences fecundity or longevity.

It is possible that the physical leaf structure of *T. occidentale* contributes to the feeding preferences, in particular that of *S. viridis*, which feeds on the mesophyll (Maclagan 1932). In comparison to *T. repens*, *T. occidentale* leaves tend to have thicker epidermal layers, with a matt upper

surface and a very glossy lower surface. However, there may be also biochemical differences that interfere with feeding cues, such as antifeedants or repellents (Smith & Clement 2012).

In conclusion, while *T. occidentale* was fed on by both pest species, it was generally less favourable for them compared to *T. repens*. This reinforces the view of Williams et al. (2009) that *T. occidentale* is a good species to investigate for resistance against pest stress. While longer duration experiments are needed to confirm that *T. occidentale* supports development and survival of all life stages, *S. lepidus* appears to be a good species to use in this work although pre-selection for adult weevils of similar size is recommended to reduce variability.

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