



## RESEARCH NOTE

# Initial test of a semiartificial diet for the thistle biocontrol beetle, *Cassida rubiginosa*

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**Summary** The thistle biocontrol beetle, *Cassida rubiginosa* is established in New Zealand, but often not sufficiently abundant to achieve control of the weed, *Cirsium arvense* (Californian thistle). Mass production of the beetle could enhance biocontrol efforts through supplemental and inundative releases. We carried out an initial test of a semiartificial diet (containing host plant material) designed for laboratory mass production of the beetle. Larval survival rates were tested on diets with three different concentrations of preservatives (full, half, and no preservative), and compared to a positive control (leaf disc of *Cirsium arvense*), and a negative control (water). Only larvae on the leaf disc developed to the adult stage. Of the diets, the longest survival time was on the full preservative diet, with a mean mortality time of  $8.8 \pm 0.6$  days, and a maximum survival time of 21 days. Although no larvae completed development on the diets, some important progress was achieved: (1) Neonate larvae were mobile on the diet; (2) larvae fed on the diet; and (3) there was adequate control of microbial contamination without being acutely toxic to the larvae. Further development of a diet for *Cassida rubiginosa* should focus on nutritional components for larval development.

**Keywords** mass-rearing, artificial diet, weeds

### INTRODUCTION

*Cassida rubiginosa* (Coleoptera: Chrysomelidae) is a leaf-feeding beetle of Eurasian origin that was introduced to New Zealand in 2007 as a biological control agent against *Cirsium arvense* (Californian thistle) (Cripps et al. 2011). The beetle completes one generation per year and both the adults and larvae feed on the leaves of many thistle species (Cardueae) from spring until autumn (October to March) (Ward & Pienkowski 1978; Cripps 2013). The primary host plant of the beetle is *Cirsium arvense*, and at high population densities, the beetle can consume all green leaf tissue, causing a 'skeletonising' effect on the weed (Cripps 2013). A controlled field evaluation of the beetle showed that at least 10 *Cassida rubiginosa* larvae per shoot was necessary to reduce the density and local spread of *Cirsium arvense* under typical sheep-grazed pasture management (Cripps et al. 2019). However, most populations of *Cassida rubiginosa* in New Zealand appear to persist at densities less than 10 larvae per shoot, with only occasional outbreak populations exceeding this density (M. Cripps, personal observation). Since *Cirsium arvense* remains one of the most economically damaging pasture weeds in New Zealand (Bourdôt et al. 2016), there is high demand for this biocontrol agent, especially for hill-country pasture where spraying herbicide is not practical or economical.

Currently, the beetle is redistributed from successful field sites, or reared on host plants in cages, and then released at field sites. Development of an artificial, or semiartificial diet, could allow for the mass production of the beetle and possibly the commercialisation of this biocontrol agent. There is a long tradition of rearing insects on artificial diets, with diets developed for many insects, including pests and specialised biocontrol agents (Singh 1977; Cohen 2015). Diets for specialised herbivorous insects are often 'semiartificial' since it is usually necessary to incorporate the host plant in the diet to obtain the chemical cues required for the insect to initiate and sustain feeding (Cohen 2015). The objective of the current study was to begin development of an artificial diet that could be used to mass rear the beetle.

### MATERIALS AND METHODS

A semiartificial diet was created based on the diet described by Blossey et al. (2000) for the purple loosestrife (*Lythrum salicaria*) biocontrol weevil, *Hylobius transversovittatus*, but modified to contain freeze-dried shoots of *Cirsium arvense* (Table 1). This diet was selected because it was a modification of a 'general purpose' diet (Singh 1983) that included host plant material for a biocontrol agent. On 11 October 2021, approximately 2 kg of fresh *Cirsium arvense* shoots (stems

**Table 1** Diet ingredients.

Ingredient	Quantity		
	Full preservative	Half preservative	No preservative
<i>Pre-autoclave</i>			
Water (mL)	188.24	188.24	188.24
Agar (Oxoid Bacteriological) (g)	6.12	6.12	6.12
Yeast extract (Bacto®) (g)	5.10	5.10	5.10
Sucrose (g)	7.54	7.54	7.54
Wesson's salts (MP Biomedicals) (g)	1.78	1.78	1.78
Casein (Sigma®) (g)	8.22	8.22	8.22
<i>Post-autoclave</i>			
Freeze-dried <i>Cirsium arvense</i> (g)	23.52	23.52	23.52
Vanderzant vitamin mix (Sigma®) (g)	2.26	2.26	2.26
Sorbic acid (g)	0.58	0.29	-
Methyl paraben (g)	0.30	0.15	-

and leaves) were collected from an established patch of plants on the AgResearch Lincoln campus. The shoots were mixed with enough water (approximately 200 mL) to blend the plant material into a smooth consistency using a kitchen blender. The blended plant material was then freeze-dried for seven days to create a dry powder. The diet was made in a two-stage process, with the diet ingredients separated into autoclaved, and non-autoclaved portions (Table 1). The toxicity of diet preservatives is one of the primary hurdles to overcome (Marrone et al. 1985; Cohen 2015) so different concentrations of the diet preservatives (sorbic acid and methyl paraben) were tested. Three diet treatments were prepared that were all the same except for the amounts of preservatives added.

The first diet treatment (Full preservative) contained the full rate of preservatives according to the recipe of Blossey et al. (2000). The second diet treatment (Half preservative) contained half the rate of preservatives, and the third diet treatment (No preservative) had no preservatives (Table 1). The post-autoclaved ingredients for each diet treatment were homogenised with the pre-autoclaved ingredients using a hand blender while the agar was still molten. While warm, approximately 10 mL of diet was dispensed into 30 mL plastic portion cups (Huhtamaki Foodservice, New Zealand) using a syringe. The prepared portion cups of diet were refrigerated at 4°C until ready for use in the experiment. In addition to the diet treatments, a positive control (Leaf) was prepared by collecting fresh *Cirsium arvense* leaves and excising leaf discs with a 12-mm diameter corkborer. The leaf discs were placed on pieces of moistened filter paper (Whatman® Number 2) cut to 25 mm diameter and placed inside the portion cups. The final treatment was a negative control (Water) in which only moistened filter paper was added to the portion cups. The portion cups for all treatments had tight-fitting lids to retain humidity.

To obtain larvae for the experiment, a breeding colony of approximately 130 adult *Cassida rubiginosa* was established from beetles collected on *Cirsium arvense* and *Cirsium vulgare* (Scotch thistle) in the Lincoln area from 22 September to

15 October 2021. The beetle colony was maintained in a laboratory at AgResearch at constant temperature (20°C) in 2-L ventilated plastic containers and fed fresh *Cirsium arvense* clippings every two to three days. Egg masses were collected from the leaves and transferred onto moistened filter paper placed inside 90-mm Petri dishes. Beginning on 26 October, individual naïve larvae were added to the portion cups for each treatment using a fine paintbrush. Larvae were applied one block at a time, and all larvae used in the experiment were applied within 24 hours. Each treatment was comprised of three replicates of 20 portion cups. The 20 portion cups for each replicate were contained within a 2-L plastic box, and the 15 boxes were arranged in a randomised block design, placed on a shelf within a growth room.

The growth room was set to a photoperiod of 18 hours light and 6 hours dark. The temperature was maintained at 22°C during the light hours (5 am to 11 pm), and the heating was turned off during the dark hours (11 pm to 5 am). This photoperiod and temperature were selected since it was reported to be optimal for the survival and development time of *Cassida rubiginosa* larvae (Kutcherov et al. 2019). In one replicate of a diet, leaf, and water treatment, an extra portion cup was included with a temperature and humidity datalogger (iButtonLink LLC, Wisconsin, USA) placed inside to precisely record the temperature and humidity conditions experienced by the larvae. The temperature and relative humidity (RH%) inside the portion cups with dataloggers were recorded every 30 minutes and the mean temperatures and RH% during the light hours and dark hours were calculated. On average, the temperature inside the portion cups was 23.3°C and 19.8°C for light and dark periods, respectively, over the duration of the experiment. Differences in temperature between the treatments was less than 1.0°C. RH% remained near 100% in all treatments throughout the experiment.

Larval observations were recorded every day for the first three days of the experiment, and then three days per week for the remainder of the experiment. The larvae were observed

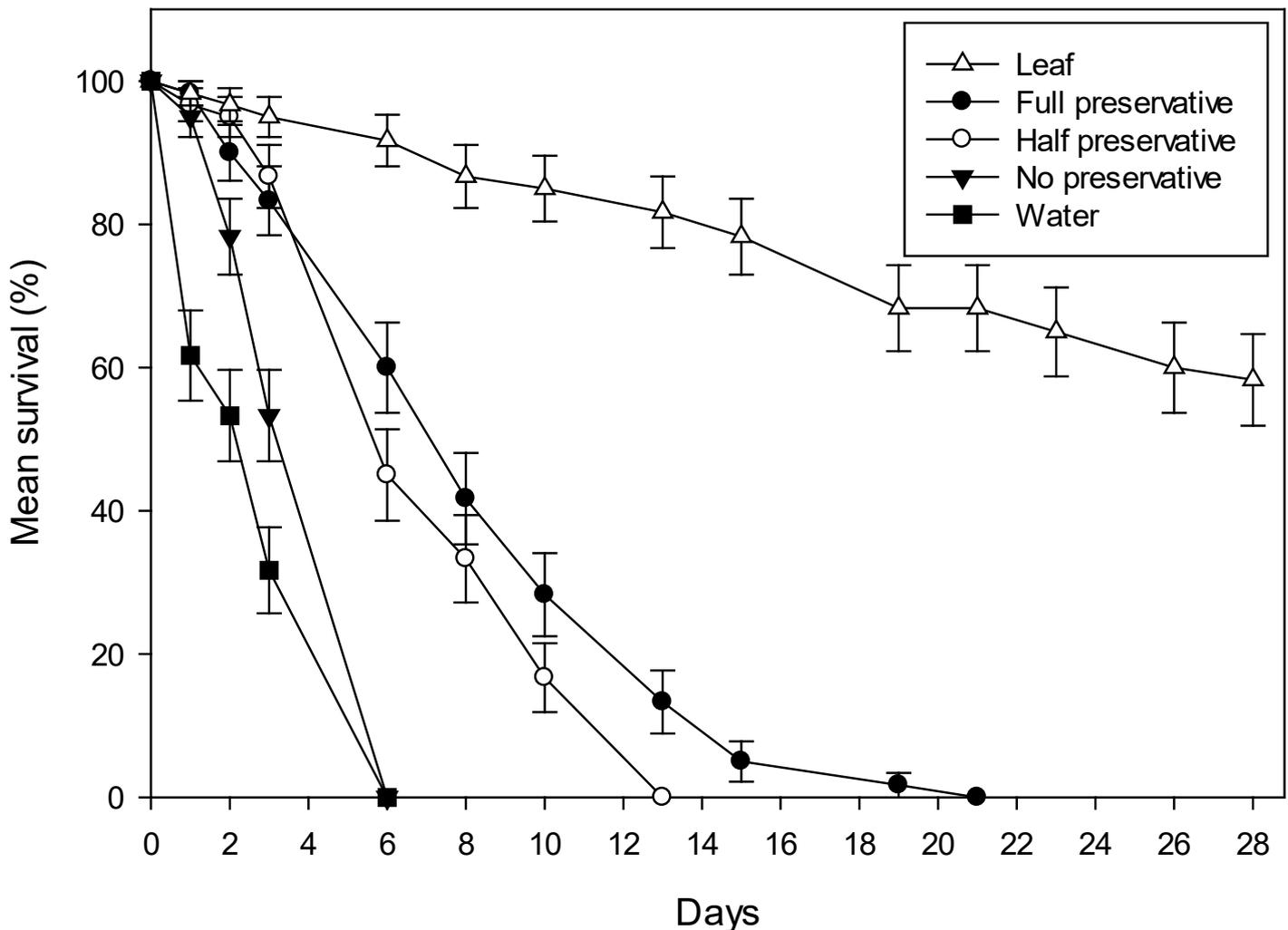
under a compound microscope and at each observation time the larval survival (determined by movement of larvae), and the presence or absence of a faecal shield, was recorded. Larvae of Cassidine beetles, including *Cassida rubiginosa*, form a 'faecal shield' on their caudal process composed of exuviae from successive moults and faecal material (Chaboo 2005). For the duration of the experiment the leaf discs in the positive control treatment were replaced once per week. For all treatments, tap water was replenished at each observation period using an eyedropper, if the media or filter paper appeared dry.

### Statistical analysis

The survival rates (%) of *Cassida rubiginosa* larvae over time were analysed using the Kaplan-Meier (K-M) method. The K-M method is a nonparametric method for analysing survival data. In the K-M analysis, the series of survival rates observed over multiple times per treatment was compared over the entire observation period of 28 days. Pair-wise comparisons were made between each of the five treatments using log-rank tests. The K-M analysis was carried out with Minitab version 16.2.2. (<https://www.minitab.com/>)

### RESULTS AND DISCUSSION

The larval survival rates were significantly different among all five treatments (Figure 1; Supplementary Table S1). The treatments with the most similar survival rates were the full and half preservative diets ( $\chi^2=3.88$ ,  $df=1$ ;  $P=0.049$ ), followed by the no preservative and water diets ( $\chi^2=9.19$ ,  $df=1$ ;  $P=0.002$ ). Pair-wise comparisons between all other treatments were similar, with highly significant differences ( $P<0.001$  for all other pair-wise comparisons). No larvae completed development on any of the semiartificial diets, or water (Figure 1). For the three diets and water, the mean ( $\pm$ SE) time (days) to death was  $8.8 \pm 0.6$ ,  $7.6 \pm 0.4$ ,  $4.3 \pm 0.2$ , and  $3.1 \pm 0.3$ , for the full, half, no preservative, and water, respectively. For the leaf treatment 58% (35/60) of larvae survived to the adult stage (Figure 1). The time for larvae on the leaf disc to reach the adult stage ranged from 21 to 28 days. This is similar to the 22.5 days reported by Kutcherov (2019) at constant 22°C, and much faster than the 47 to 68 days when reared on live plants in ambient outdoor conditions (Cripps et al. 2015). Of the 42% (25/60) of larvae that died on the leaf disc, their death occurred on average at  $15 \pm 1.6$  days.



**Figure 1** Mean ( $\pm$ SE) percent survival of *Cassida rubiginosa* larvae on three semiartificial diets (full, half, and no preservative), a *Cirsium arvense* leaf disc, or water.

Although no larvae successfully completed development on the diet treatments, there were indications that *Cassida rubiginosa* is likely amenable to rearing on a semiartificial diet. On this diet, neonate larvae were observed to move freely on the surface. This is important because if a diet is too wet or sticky, larvae can become entangled in the media causing high mortality (Moore & Navon 1974; Rabab et al. 2016). A viscous or sticky texture can be caused by an unsuitable or low concentration of a gelling agent, or a formulation with high moisture content, or oily ingredients, as reported by Jin et al. (2018). These authors designed an artificial diet for the coconut black-headed caterpillar, *Opisina arenosella*, and concluded that linseed oil made the diet viscous and sticky, causing mortality of small, early instar, larvae. All physical properties of artificial diets such as texture, hardness, homogeneity, and water content, are critical for the mobility of larvae, and regulated by the addition of bulking and gelling agents, such as cellulose, agar, and polysaccharide gums (Vanderzant 1969; Lingappa 1987; Vera et al. 2007). For the diet tested in this experiment, the concentrations of agar and freeze-dried plant material provided a firm, non-sticky, surface suitable for the movement of *Cassida rubiginosa* larvae.

The development of a faecal shield was observed for all larvae on the three diets, and the leaf disc, but not in the water control. The formation of faecal shields indicates that the larvae received the necessary cues to initiate feeding. In contrast, the caudal processes of all larvae on the water treatment remained free of exuviae and faecal matter. With only water, most larvae died within 3.1 days, compared to 8.8 days on the full preservative diet (Figure 1). Decreasing the concentration of preservatives did not improve the survival rate. In fact, survival rates were slightly lower on the half preservative compared to the full preservative treatment, suggesting that it was not the toxicity of preservatives inhibiting development. However, without preservatives, the diet became completely contaminated with fungal growth after six days and no larvae survived beyond this time (Figure 1). There was no visual evidence of fungal or bacterial contamination in either the full or half rate preservative diets, indicating that this component of the diet is adequate at even the half rate. Antibiotics are commonly used in artificial diets to prevent bacterial growth. However, they were not included in this trial since *Cassida rubiginosa* has a symbiotic bacterium that aids in digestion of pectin (Salem et al. 2017).

The failure of larvae to develop was likely due to the absence of essential nutrients, most notably cholesterol. Cholesterol is the precursor for the synthesis of ecdysteroids, the hormones involved in the moulting process (Klowden 2002). Therefore cholesterol is often included in insect diets to achieve larval moulting and development (Cohen 2015). Another nutritional consideration is the concentration of sucrose in the diet. Sugar is typically required as an energy source but some insects are sensitive to the concentration of this ingredient. Improvements to the diet for the purple loosestrife biocontrol weevil showed that sucrose concentrations over 2% caused an exponential decline in survival (Tomic-Carruthers 2007). The sucrose concentration in the diets tested here for *Cassida rubiginosa*

was approximately 3%, and therefore future diets should be tested with reduced sucrose.

Finally, the rearing conditions would likely be improved with lower RH% inside the portion cups. The RH% was near 100%, which is generally too high for most insects, since this can promote disease, and potentially cause drowning (Singh 1982). This may have contributed to the high larval mortality on the leaf disc and on the semiartificial diet, and therefore ventilating the rearing containers would likely be beneficial.

## CONCLUSIONS

This study provides a proof of concept that a semiartificial diet can be developed for *Cassida rubiginosa*. This initial diet achieved: (1) a suitable texture for mobility of neonate larvae; (2) evidence of feeding; and (3) sufficient control of microbial contamination for at least 28 days without an acutely toxic effect on the larvae. Further development of a diet for *C. rubiginosa* should focus on the nutritional components critical for larval development, such as cholesterol. Ultimately, an efficient and low-cost diet for rearing *C. rubiginosa* could enhance biocontrol efforts through supplemental and inundative releases.

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## SUPPLEMENTAL TABLE

**Table S1** Results of pair-wise comparisons of survival rates (%) of *Cassida rubiginosa* larvae between treatments (leaf disc of *Cirsium arvense*, full preservative diet, half preservative diet, no preservative diet, or water) over the 28-day duration of the experiment. Survival rates were analysed using the Kaplan-Meier method.

Compared treatments		Chi-squared ( $\chi^2$ ) statistic	Df	P-value
Full preservative		79.18	1	< 0.001
Half preservative		85.60	1	< 0.001
No preservative	vs. Leaf	93.74	1	< 0.001
Water		106.44	1	< 0.001
Half preservative		3.88	1	0.049
No preservative	vs. Full preservative	43.68	1	< 0.001
Water		60.29	1	< 0.001
No preservative		37.39	1	< 0.001
Water	vs. Half preservative	55.68	1	< 0.001
Water	vs. No preservative	9.19	1	0.002