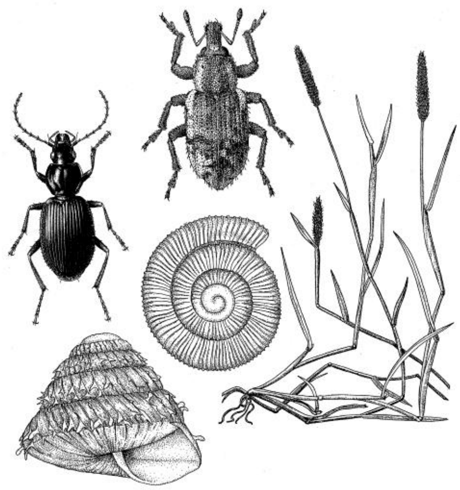
Effects *of Neotyphodium uncinatum* infected, loline-containing, meadow fescue–ryegrass hybrid grasses on the feeding behaviour of Orthoptera.

3. Assessment of response of wingless grasshopper (*Phaulacridium vittatum*) (Acrididae) to artificial diets and seed containing loline alkaloids

Report prepared for Cropmark Seeds Ltd

by Gary M. Barker



G. M. Barker & Research Associates

The Invertebrate Biodiversity Specialists
Working in production agriculture and its interface with biodiversity conservation

Gary M. & Maria P. Barker
As Trustees for *The G & M Barker Family Trust*, 125 Charltons Road, Stony Creek, Victoria 3957, Australia. Tel. +61 3 5689 1236 slug20@bigpond.com

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SUMMARY

Two laboratory experiments were conducted to determine the response of the wingless grasshopper (*Phaulacridium vittatum*) to loline alkaloids in their diet.

Experiment 1 examined a gradient of loline alkaloids in artificial diet. Visual scoring of extent of feeding on the diet plugs, and measured quantities of diet consumed, indicated statistically significant treatment differences in grasshopper feeding. Regression analyses showed that feeding, indicated by both visual scores and quantities of diet consumed, was strongly related to concentration of lolines in the diet. The amount consumed by grasshoppers on diet containing 5600 μg/g lolines was only 8% relative to those on loline-free diet.

In Experiment 2, grasshopper feeding on loline-containing seed was compared to grasshopper feeding on endophyte-free (loline-free) seed. By 36 hours, grasshopper had destroyed 36% of endophyte-free but had destroyed only 12% of loline-containing seed.

Gary M. & Maria P. Barker
As Trustees for *The G & M Barker Family Trust*, 125 Charltons Road, Stony Creek, Victoria 3957, Australia. Tel. +61 3 5689 1236 slug20@bigpond.com

INTRODUCTION

Because of their natural role in biological protection of the grass hosts, *Neotyphodium* endophytes are widely recognised as beneficial mycosymbionts in pastoral and turf systems. There is considerable interest internationally in the development of forage and turf grasses infected with *Neotyphodium* endophytes. Understanding the role of different alkaloids in protecting plants against various herbivorous pests is critical to development of endophyte-containing grasses for commercial use.

Cropmark Seeds Ltd. has been developing forage grasses based on Meadow fescue (*Festuca pratensis*) and its loline-producing endophyte *Neotyphodium uncinatum* because of potential agronomic advantages, not least pest resistance and tolerance. Genotypes infected with *N. uncinatum* has been shown to deter attack from several pasture insects, as summarised in the first part of this report series to Cropmark Seeds Ltd (Barker, 2011). Among other attributes, Cropmark Seeds is particularly interested in the agronomic advantage of meadow fescue and hybrid genotypes in regions prone to pest outbreaks. Crickets and grasshoppers are major pasture pests in many parts of Australia and northern New Zealand, but endophyte effects on these insects are poorly known.

This report describes two laboratory experiments, one with with loline-containing artificial diets, the other with loline-free and loline-containing seed, to determine the response of Wingless grasshopper (*Phaulacridium vittatum* (Sjöstedt, 1920)) (Acrididae) to loline alkaloids in their diet.

Gary M. & Maria P. Barker
As Trustees for *The G & M Barker Family Trust*, 125 Charltons Road, Stony Creek, Victoria 3957, Australia. Tel. +61 3 5689 1236 slug20@bigpond.com

MATERIALS AND METHODS

Experiment 1. An assay was performed using artificial diets containing a range of loline concentrations. In the absence of the availability of analytical grade lolines, the diets were prepared by incorporating different ratios of loline-containing (*Neotyphodium uncinatum*-infected) and loline-free (endophyte-free) grass seed into the agar-based diets. Loline-containing seed (*Festulolium* FHCF0802U2, 2348M, 26514 µg/g lolines) and loline-free seed (Ultra *Festulolium* URL10L1) were each finely ground in a domestic coffee grinder and added in varying ratios to molten (50 °C) 4% agar in tap water to yield seven treatments varying in loline concentration (Table 1). The molten diet thus prepared was poured into Petri dishes and held at 4 °C to solidify.

The ~25% dry matter content of the diet approximates well that occurring in summer grass forage material which are the grasshoppers' usual adult diet in the field.

Table 1. Experiment 1: Artificial diets made from mixing into 4% agar different ratios of finely ground loline-containing (*Neotyphodium uncinatum*-infected) (26514 µg/g total lolines) and loline-free (endophyte-free) grass seed.

Treatment	Loline concentration in diet (µg/g)	Weight of loline-containing seed (g/100g diet)	Weight of loline-free seed (g/100g diet)
1	5600	21.20	0
2	2800	10.60	10.60
3	1400	5.30	15.90
4	700	2.65	18.55
5	350	1.32	19.88
6	175	0.66	20.54
7	0	0.00	21.20

Adult Wingless grasshoppers were collected from pastures near Yarrum, southeastern Victoria, Australia and held for 24 hours without food before use in the experiment. Only healthy grasshoppers were used. Each replicate comprised five grasshoppers placed in a 500ml plastic jar, randomly assigned to treatment, and provided with two pre-weighted plug of diet taken from the appropriate stock diet with a 10mm cork borer. Each treatment had five replicates, arranged on the laboratory bench in a randomised block design. To maintain humidity, the jars were provided with moistened filter paper and covered with a plastic press-on lid.

Additional diet plugs were held in jars as described above, but without grasshoppers, to determine weight loss in the absence of insect feeding. This weight loss was used as a correction factor in estimates of amount of diet consumed by grasshoppers during the experiment.

After 12 and 36 hours the extent of feeding on the diet plugs was assessed visually on a scale of 0 to 10 (0, no feeding evident; 1 = minimal evidence of feeding and minimal production of faeces; 10 = diet plugs extensively shredded and extensive faecal production). At 36 hours the residual diet plugs were weighed. The amount of diet consumed was estimated was 'initial diet weight' minus 'final diet weight' corrected for diet weight loss in the absence of grasshoppers.

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Data on visual scores of feeding on the diet, and on estimates of amounts of diet consumed, were subject to analysis of variance. In fitting regressions, the data on the y-axis (feeding scores) were transformed Ln (x) to provide the best description of the relationship between loline concentrations and grasshopper feeding. These statistical analyses were performed using S-Plus.

Experiment 2. An assay was performed using seed of loline-containing seed (*Festulolium* FHCF0802U2, 2348M, 26514 µg/g lolines) and loline-free seed (Ultra *Festulolium* URL10L1).

Wingless grasshoppers collected from pastures at Yarrum, southeastern Victoria, Australia were held for 24 hours without food before use in the experiment. Only healthy grasshoppers were used. Each replicate comprised 5 grasshoppers, randomly assigned to treatment, placed in a 500ml plastic jar and provided with 20 seeds - either loline-containing or loline-free. Each treatment had five replicates, arranged on the laboratory bench in a randomised block design. To maintain humidity, the jars were provided with moistened filter paper and covered with a plastic press-on lid. The offered seeds did not come into contact with the moistened filter paper and thus germination was not initiated during the course of the experiment.

After 12, and 36 hours, the extent of feeding on the seed was assessed visually by counting the number of seeds damaged by grasshoppers.

Data on numbers of seeds damaged by grasshoppers were subject to analysis of variance using S-Plus.

Gary M. & Maria P. Barker
As Trustees for *The G & M Barker Family Trust*, 125 Charltons Road, Stony Creek, Victoria 3957, Australia. Tel. +61 3 5689 1236 slug20@bigpond.com

RESULTS

Experiment 1. Visual scoring of extent of feeding on the diet plugs indicated statistically significant treatment differences in grasshopper feeding that was more-or-less consistent between the 12 and 36 hour assessments (Table 2). The extent of feeding, as indicated by these visual scores, was strongly related to the loline concentration in the diet (Figure 2), suggesting that the loline suppressive effect on feeding developed rapidly at the lower end of the loline concentration gradient and additional suppression at the higher end of the gradient was modest.

Table 2. Experiment 1: Visual scores of extent of feeding by Wingless grasshoppers on agar-based diets containing varying concentrations of loline alkaloids.

Treatment	**Loline concentration (μg/g)**	**Visual scores of feeding (0-10 scale)**[1]	
		12 hrs	**36 hrs**
1	5600	2.8	3.4
2	2800	3.2	4.2
3	1400	3.2	4.8
4	700	4.2	5.8
5	350	4.8	5.8
6	175	5.8	6.4
7	0	6.2	7.0
Mean		4.31	5.34
Treatment effects			
$F_{6, 34}$		9.833	7.160
P value		<0.001	<0.001
Fisher's $LSD_{0.05}$		1.555	1.428

[1.] Results presented as cumulative scores over the 36 hour experimental period.

Quantities of diet consumed by grasshoppers varied significantly between treatments (Table 3) and were strongly related to the loline concentration in the diet (Figure 3).

Feeding on the diet occurred at the highest loline concentration, albeit minimally. It is thus evident that the suppressive effect of lolines on feeding by grasshoppers was not absolute. The amount consumed by grasshoppers on diet containing 5600 μg/g lolines was only 8% relative to those on loline-free diet after 36 hours.

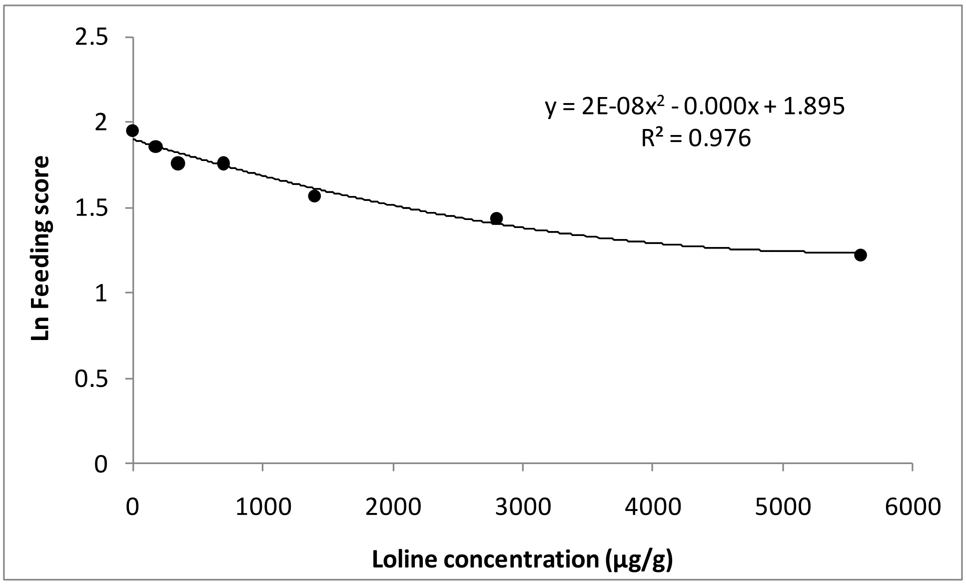

Figure 2. Experiment 1: Relationship between loline concentration in the diet (natural log scale) and visual scores of feeding by Wingless grasshoppers on diet plugs at 36 hours.

Table 3. Experiment 1: Quantities of on agar-based diet, containing varying concentrations of loline alkaloids, consumed by Wingless grasshoppers.

Treatment	Loline concentration (µg/g)	Diet consumed (mg).	
		12 hours	36 hours
1	5600	8.64	13.10
2	2800	10.64	33.64
3	1400	15.22	42.66
4	700	19.60	63.60
5	350	30.48	86.84
6	175	36.68	118.76
7	0	43.56	152.60
Mean		23.546	73.028
Treatment effects			
$F_{6, 34}$		99.599	178.361
P value		< 0.001	< 0.001
Fisher's $LSD_{0.05}$		4.920	12.988

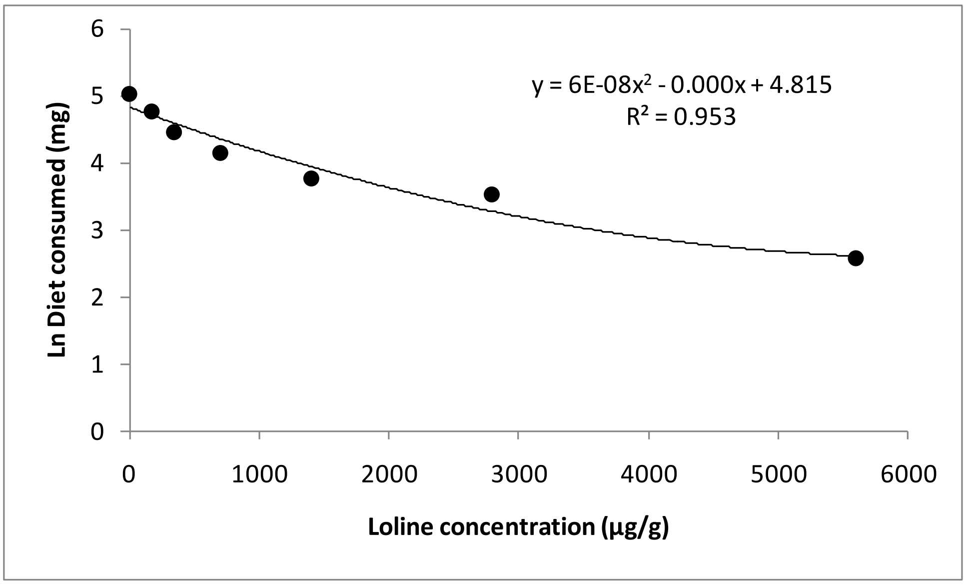

Figure 3. Experiment 1: Relationship between loline concentration in the diet and quantity of diet consumed by Wingless grasshoppers at 36 hours.

Experiment 2. At 36 hours, grasshoppers had destroyed on average 7.2 of 20 seeds on offer in the endophyte-free treatment but had feed on only 2.4 seeds in the endophyte-infected treatment ($F_{1, 9} = 46.080$, $P < 0.001$).

Gary M. & Maria P. Barker
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DISCUSSION

These experiments indicate that loline alkaloids are a strong feeding deterrent in the adult stage of the Wingless grasshopper. Young hoppers feed on prostate dicotyledonous plants (such as clovers and flat weeds in pastures) rather than grasses, so the results obtained in this work relate only to the adult stage.

Gary M. & Maria P. Barker
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Gary M. & Maria P. Barker
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