

# Mice find endophyte-infected seed of tall fescue unpalatable – implications for the aviation industry

S. C. Finch\*, C. G. L. Pennell†, J. W. F. Kerby\* and V. M. Cave\*

\*Ruakura Research Centre, AgResearch Limited, Hamilton, New Zealand, †Lincoln Research Centre, AgResearch Limited, Christchurch, New Zealand

## Abstract

Bird strike is a significant problem for the aviation industry, caused in part by the attractiveness of the grass surrounding airports to birds. Endophyte-infected grasses such as Avanex<sup>®</sup> have been shown to reduce bird populations at airports through the production of secondary metabolites. These metabolites are unpalatable to herbivorous, omnivorous and granivorous birds and are known to reduce insect densities, thereby making the sown areas less attractive to insectivorous birds. Raptors also provide a bird-strike threat which could be reduced by controlling mouse populations around airports. In this study, the effect of endophyte-infected seed on mouse feeding behaviour has been investigated. By performing a choice trial offering diets containing endophyte-infected and endophyte-free tall fescue seed, it was shown that endophyte-infected seed was less palatable to mice ( $P < 0.001$  in a 14-d trial). Furthermore, when diet positions were reversed, mouse feeding behaviour was modified to again select endophyte-free material ( $P < 0.001$  over a further 14 d). This result shows that endophyte-infected grasses could not only be used in the control of bird populations but they also have the potential to control mouse populations which in turn would decrease the number of raptors. This use of endophyte-infected grasses in areas surrounding airport runways shows great promise for the aviation industry.

**Keywords:** Avanex<sup>®</sup>, bird strike, ergovaline, *Epichloë coenophiala*, wildlife control

## Introduction

Passenger air traffic and aircraft flight routes have increased substantially since 1980 (Sodhi, 2002). This has led to an increasing number of documented bird strikes involving aircraft with 10 343 incidents recorded throughout the USA in 2012 (Dolbeer *et al.*, 2013) and with a financial cost estimated at US\$2 billion per year (Pinos, 1996). Data collected by the International Civil Aviation Organisation has shown that most bird strikes occur at aircraft take-off and landing phases (Cleary *et al.*, 2005). It is also recognized that on many airfields, the attraction for bird species is the grass itself for herbivores such as Canada geese (*Branta canadensis*), the seeds for granivorous birds [such as greenfinches (*Carduelis chloris*)], insects for insectivorous birds [such as starlings (*Sturnus vulgaris*) and gulls (*Larus dominicus*)] or small mammals, the prey of raptors. Therefore, bird strikes could be alleviated by reducing the number of birds at and near airfields, especially the runways, by reducing the attractiveness of the grass to birds and rodents.

In the past, the aviation industry has relied on 'primary response mechanisms' such as acoustical distress calls, gas guns, lights, lasers, dogs, falconry, kites, balloons, sticky pastes, spikes, wires and netting which all attempt to frighten birds from an area. However, these 'primary repellents' are costly to maintain and do not reduce the attractiveness of the area to bird visits. Other tools described as 'secondary repellents' include chemical irritants or taste aversion sprays which act as feeding deterrents to grass- and seed-eating birds. These agents are known to be more effective in the long term and have been described as causing 'post-digestion feedback' (Conover and Messmer, 1996b), a learned response that alters feeding behaviour. Habitat modification is seen as the most effective long-term solution to reducing wildlife problems at airports (Blackwell *et al.*, 2009; DeVault *et al.*, 2011) with many universities and other institutions now undertaking research in wildlife management and

Correspondence to: S. C. Finch, Ruakura Research Centre, AgResearch Limited, Hamilton, PB 3123, New Zealand.  
E-mail: sarah.finch@agresearch.co.nz

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many airport authorities now implementing a proactive wildlife hazard management plan.

Previous studies have investigated the use of grasses infected with selected fungal endophytes that express endophyte-produced metabolites for controlling birds (Pennell and Rolston, 2003, 2011, 2013; Pennell *et al.*, 2010). A study measuring the effect of grass–endophyte associations on Canada geese showed that geese consumed up to four times more perennial ryegrass (*Lolium perenne* L.) containing no endophyte than that containing the endophyte *Epichloë festucae* var. *lolii* [formerly known as *Neotyphodium lolii* (Latch, Christensen & Samuels) (Leuchtman *et al.*, 2014)]. Furthermore, the feeding behaviour of the geese was modified over time, suggesting a post-digestion feedback response. This effect was thought to be due to the presence of the endophyte-expressed metabolite, ergovaline (Pennell and Rolston, 2003). Similarly, another study comparing tall fescue (*Festuca arundinacea* Shreb.) infected with the endophytic fungus *Epichloë coenophiala* [formerly known as *Neotyphodium coenophialum* (Morgan-Jones & W. Gams)] with endophyte-free grass showed that Canada geese developed a preference for the endophyte-free material (Conover and Messmer, 1996b). An effect of endophytes on seed-eating birds has also been demonstrated; Conover and Messmer (1996a) showed that zebra finches could discriminate between tall fescue seeds infected with the endophytic fungus *E. coenophiala* and those without endophyte after 4 months of feeding and chose to consume a greater quantity of the endophyte-free material.

Endophytes have long been associated with insect deterrence due to the expression of a range of secondary metabolites. In perennial ryegrass infected with *E. festucae* var. *lolii*, peramine is responsible for insect resistance (Rowan and Gaynor, 1986; Rowan *et al.*, 1986). In tall fescue infected with the endophytic fungus *E. coenophiala*, lolines are responsible for resistance to a range of insect pests (Reidell *et al.*, 1991; Bacon, 1995). In addition, ergovaline, which is expressed in many perennial ryegrass and tall fescue endophyte associations, provides resistance to insects such as African black beetle (*Heteronychus arator*) (Ball *et al.*, 1997). These effects on insects could be of benefit to the aviation industry as a reduction in insect numbers in areas surrounding airports could potentially result in a lower population density of insectivorous birds.

Considerable research has been conducted to investigate the secondary metabolites expressed by different *Epichloë* endophyte strains. In addition to producing secondary metabolites responsible for insect resistance, endophytes also produce metabolites responsible for animal health problems such as the lolitremes from

perennial ryegrass (Fletcher and Harvey, 1981; Gallagher *et al.*, 1981; Miles *et al.*, 1994; Munday-Finch *et al.*, 1995, 1996, 1997, 1998; Gatenby *et al.*, 1999) and ergovaline from tall fescue (Schmidt *et al.*, 1982; Ball *et al.*, 1997; Fletcher, 1999). For this reason, most research to date has been focussed on identifying endophyte strains that express the agriculturally favourable metabolites without the detrimental ones to yield an endophyte strain that is less toxic to livestock but resistant to insect pests. The transfer of these selected endophytes into modern improved grass cultivars, using the methods of Latch and Christensen (1985), has resulted in a number of successful commercial forage products that are of great benefit to the New Zealand and USA economies (Johnson *et al.*, 2013). More recently, however, it has been recognized that some endophyte strains not suitable in pastoral grazing situations could be developed for the aviation and recreation industries to control wildlife.

Establishment of novel endophyte-infected grasses for bird management at airports is now a reality with PGG Wrightson Turf (Christchurch, New Zealand), a division of PGG Wrightson Seeds Ltd, being licensed the technology for commercial production, marketing and sale. This material, branded Avanex<sup>®</sup> Unique Endophyte Technology, is now established in large areas at two international airports in New Zealand, and trials are underway in Australia (three sites) and the USA. Avanex<sup>®</sup> is the result of over 12 years research and development by scientists at AgResearch that has resulted in a product suited to the aviation industry (Pennell *et al.*, 2010) which produces both loline alkaloids (suitable for insect resistance) and ergovaline (to induce bird avoidance behaviour).

While the positive results of Avanex<sup>®</sup> on insect and bird numbers are now well documented, it is also possible that this endophyte–grass material could be utilized to control other wildlife. Many airports throughout the world have issues with raptor strikes (Caister, 2009). As raptors are attracted to grasslands by the presence of small mammals, a decrease in the numbers of these species would reduce the prevalence of raptor strikes. Filipov *et al.* (1998) showed that endophyte-infected (*N. coenophialum* = *E. coenophiala*) tall fescue reduced food intake and weight gain of rabbits compared to endophyte-free controls. Later work by Panaccione *et al.* (2006) using perennial ryegrass–*E. festucae* var. *lolii* sp. Lp1 symbiota with altered ergot alkaloid profiles showed that ergovaline is particularly important for appetite suppression in rabbits. Furthermore, C. G. L. Pennell (unpubl. results) showed a significant reduction in food intake by domestic rabbits fed grass pellets derived from the ergovaline-producing Avanex<sup>®</sup> grasses when compared with endophyte-free equivalents. Diminished population densities of four

small mammal species [eastern harvest mice (*Reithrodontomys humulis* Giglioli), short-tailed shrews (*Blarina brevicauda* Gray), pine voles (*Microtus pinetorum* McMurtree) and cotton rats (*Sigmodon hispidus* Say & Ord)] have been observed in plots of tall fescue infected with the endophytic fungus *E. coenophiala* compared with endophyte-free plots (Coley *et al.*, 1995). Previous studies have also shown effects of endophyte-infected (*E. coenophiala*) tall fescue on rodents. These effects include reduced food consumption in rats (Jackson Jr *et al.*, 1986; Varney *et al.*, 1988) and reduced reproductive potential in rats (Varney *et al.*, 1987, 1988) and mice (Zavos *et al.*, 1988, 1990).

The objectives of this study were to investigate the effect of endophyte-infected tall fescue on the feeding of mice by performing a choice trial. As mice feed on grass seed, but do not digest vegetative grass tissues, diets containing endophyte-infected (AR601) and endophyte-free tall fescue seed were prepared and fed to mice.

## Materials and methods

### Animals

Swiss albino mice (4–5 weeks of age) were used in this study. Mice were housed in pairs. In Trial 1, four cages of male and female mice were used (sixteen mice in total), and in Trial 2, six cages of male and female mice were used (twenty-four mice in total). The cages of mice were randomly allocated across three storage shelves such that the four different cage configurations (gender by diet location) were evenly represented on each shelf. Animals were housed in a temperature-controlled room ( $21 \pm 1^\circ\text{C}$ ) with a 12-h light–dark cycle. Food was replenished every second day, and animals were allowed access to food and water *ad lib*. All animal manipulations were approved by the Ruakura Animal Ethics Committee established under the Animal Protection (code of ethical conduct) Regulations Act, 1987 (New Zealand).

### Diet preparation

Regular mouse food (meat-free rat and mouse diet; Specialty Feeds, Glen Forrest, WA, Australia) was ground to a fine flour using an Udy cyclone sample mill (Udy Corporation, Fort Collins, CO, USA). Tall fescue seed (cv. Jackal) infected with endophyte strain AR601 (E+) (marketed as Avanex<sup>®</sup>; PGG Wrightson Turf and a registered trademark of PGG Wrightson Seeds) and tall fescue seed (cv. Advance) containing no endophyte (E–) were ground in the same way. Seed-containing diets were prepared by mixing seed

(E+ or E–, 150 g) with ground mouse food (350 g) using a cake mixer. Aliquots (100 g) were mixed with water (95 mL) to form a paste. This was then divided into fifteen evenly sized balls which were flattened and dried in a fan oven ( $50^\circ\text{C}$ , 24 h). This resulted in a diet with an average moisture content of 11%.

## Trial design

### Trial 1

Regular mouse cages were used in this experiment, whereby the drink bottle in each cage sat to the right of the overhead food hopper which was partitioned to allow the two diet choices to be kept separate. Due to this orientation, one diet choice was situated closer to the drink bottle than the other. To ensure that no bias was introduced due to the relative proximity to the drink bottle, the number of cages with either E+ or E– diet situated closest to the drink bottle was the same. Food consumption of each diet type was measured every 2 d and the body weight of each animal measured twice weekly. The duration of the experiment was 14 d.

### Trial 2

In this trial, mouse cages were modified such that the water bottle could be placed in the centre of the overhead food hopper with the two diet choices on either side. To ensure no bias was introduced, the number of cages with each diet on the left and right sides of the drink bottle was the same. Food consumption of each diet type per cage was measured every 2 d and the body weight of each animal measured twice weekly. After a period of 14 d, the positions of the diet choices were reversed for each cage and the trial continued for a further 14 d (i.e. cages 1, 2 and 3 had the E– diet on the left-hand side of the drink bottle for the first 14-d period and on the right-hand side of the drink bottle for the second 14-d period).

## Measurement of alkaloid levels in seed

Ergovaline was extracted from ground seed (50 mg) with prepared extraction solvent (1 mL, 50% methanol with  $0.54 \text{ ng mL}^{-1}$  ergotamine tartrate as an internal standard) for 1 h. The sample was then centrifuged (8000 g, 5 min), and a 500  $\mu\text{L}$  aliquot of the supernatant transferred to an HPLC vial for analysis. HPLC was performed using a 2.0 mm  $\times$  150 mm Gemini-NX column (3  $\mu\text{m}$  110A; Phenomenex, Torrance, CA, USA) eluted with a binary gradient as follows: 0 min, 85% (v/v) solvent A, 15% (v/v) solvent B; 0.5 min, 75% (v/v) solvent A, 25% (v/v) solvent B;

7.5 min, 40% (v/v) solvent A, 60% (v/v) solvent B; 8 min, 16.7% (v/v) solvent A, 83.3% (v/v) solvent B; 9 min, 16.7% (v/v) solvent A, 83.3% (v/v) solvent B; 9.5 min, 85% (v/v) solvent A, 15% (v/v) solvent B. Solvent A was acetonitrile–aqueous ammonium carbonate (10 mM) (1:4 v/v); solvent B was acetonitrile. The flow rate was 0.2 mL min<sup>-1</sup>. Eluting compounds were detected with a Shimadzu RF-10Axl fluorescence detector (excitation at 310 nm, emission at 410 nm; Shimadzu Corporation, Tokyo, Japan). The limit of quantification of this technique was 0.1 µg g<sup>-1</sup>.

## Statistical analysis

### Trial 1

For each cage, the total amount (in grams) of E+ and E- diet consumed over the duration of the study was calculated. An ANOVA, blocked by cage, was used to test whether the average amount of food consumed differed according to diet (E+ vs. E-), gender or proximity to the drink bottle (i.e. diet directly beside drink bottle or not).

### Trial 2

For each cage, the total amount of E+ and E- diet consumed was calculated from day 0 to day 14 and from day 14 to day 28 (after the position of the diet had been exchanged). An ANOVA, blocked by cage, was used to test whether the average amount of food consumed differed according to diet (E+ vs. E-) or gender. Data from the two time periods (days 0–14 and days 14–28) were analysed separately.

All analyses were conducted using GenStat (15th edition; VSN International Ltd., Hemel Hempstead, UK).

## Results

Analysis of tall fescue seed infected with the endophyte strain AR601 showed it to contain 7.8 µg g<sup>-1</sup> ergovaline, and the concentrations fed to mice were therefore 2.34 µg g<sup>-1</sup>. No ergovaline was detected in tall fescue seed without endophyte.

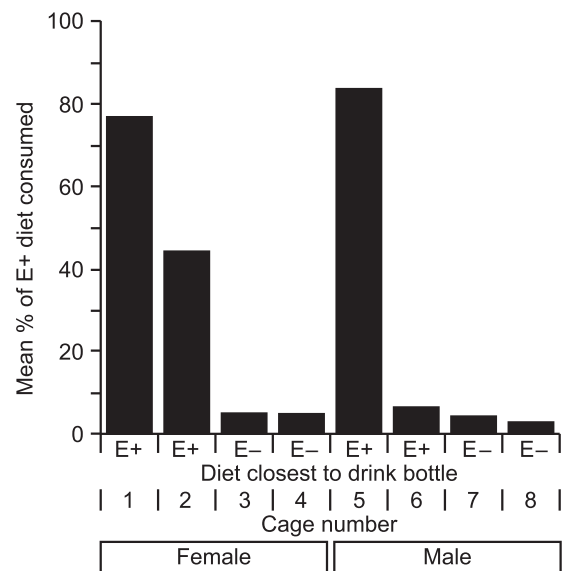
### Trial 1

All mice gained weight and were healthy throughout the experiment. There was no evidence of any interactions between diet, gender and proximity of diet to the drink bottle. Food consumption data showed that, overall, the mice ate more of the diet containing E- seed (mean consumption = 129 g total) compared to that containing E+ seed (mean consumption = 49 g

total) (sed = 33.9 g,  $P = 0.055$ ). For each cage, the amount of E+ diet consumed, expressed as a percentage of the total weight of food consumed, was calculated over the 14-d experiment and plotted (Figure 1). This revealed that in three cages (numbers 1, 2 and 5), mice ate more E+ seed diet in comparison with the other five cages which clearly ate predominately diet containing E- seed. Interestingly, these three cages all had the E+ seed diet closest to the drink bottle, suggesting that feeding behaviour may be influenced by the position of the drink bottle. This is supported by comparing the total amount of diet consumed from each diet position (mean consumption of diet closest to drink bottle = 132 g total and mean consumption of diet furthest from drink bottle = 46 g, sed = 33.9 g,  $P = 0.044$ ). As the experiment was designed to contain an equal number of cages with each diet choice in each feeding position, no bias was present but any effect of the E+ seed diet on mouse feeding behaviour would have been diluted by the effect of the water bottle position. For this reason, a second trial was performed whereby the water bottle was in a central position with the two diet choices on either side.

### Trial 2

All mice gained weight and remained healthy throughout the experiment. Food consumption data showed that over the first 14 d of the experiment, mice ate on average a total of 157 g of the diet containing E- seed and a total of 22 g of the diet containing



**Figure 1** Mean percentage of E+ diet consumed for the eight cages of mice fed E+ vs. E- treatment diets in Trial 1.

E+ seed. The effect of diet type was highly statistically significant (sed = 20.9 g,  $P < 0.001$ ). After the initial 14-d period, the position of each diet type was exchanged so that mice had to alter their feeding behaviour to continue eating their preferred diet choice. On days 14–28, mice, on average, ate a total of 130 g of the diet containing E– seed and a total of 23 g of the diet containing E+ seed. Again, this effect was highly statistically significant (sed = 13.4 g,  $P < 0.001$ ). It is interesting to consider the feeding behaviour of mice over time (Figure 2). This shows that over the first two experimental days, mice generally did not discriminate between the two different diet choices, although female mice ate predominantly diet containing E– seed from the beginning. By days 2–4, however, mice had modified their feeding behaviour to eat less of the diet containing E+ seed. Similarly, on days 14–16, after the positions of the diet choices had been reversed, more diet containing E+ seed was initially consumed before feeding behaviour was modified by days 16–18.

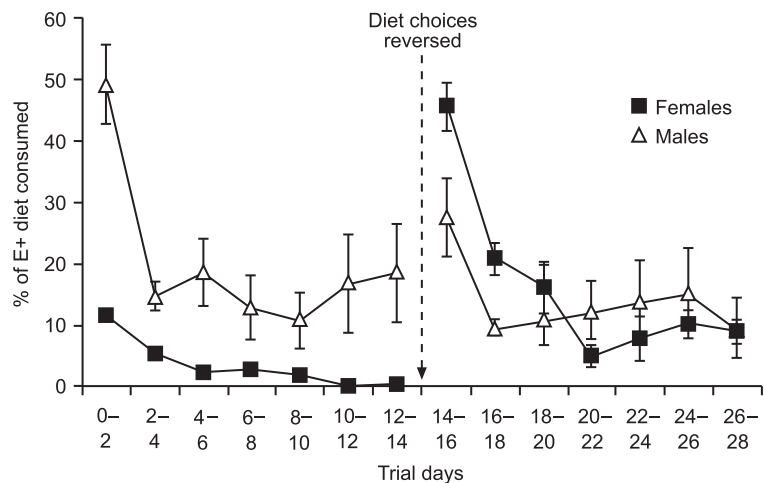
## Discussion

The results of Trial 1 suggest that, given the choice, mice would avoid eating diet containing endophyte-infected material. However, the results of this trial were complicated by an effect of placement of the water bottle, with mice appearing to consume more of the diet choice located closest to the drink bottle. We are unaware of any previous studies that have shown this effect, and it should be taken into account when planning trial designs for diet-choice experiments.

In Trial 2, the drink bottle was placed between the two different diet choices to eliminate the drink bottle placement effect. Results of this trial very clearly

demonstrated that mice preferred to eat diet containing E– seed in comparison with that containing E+ seed. At the start of the experiment and after the positions of each diet choice had been reversed (at day 14), mice consumed more even quantities of the two diet types but within 2–4 d their feeding behaviour was modified to consume predominately diet containing E– seed.

This experiment showed that mice find E+ seed less palatable than E– seed which is likely to be due to the presence of endophyte-produced secondary metabolites, including ergovaline. Although it is difficult to extrapolate the result of a tightly controlled experiment to the natural environment, the very strong observed effect would suggest that the sowing of *Avanex*<sup>®</sup> at airports has the potential to control mouse populations. Consistent with this hypothesis is the general observation made by farm staff during seed harvesting that far fewer mice are present in *Avanex*<sup>®</sup> pastures compared to other cultivars (S. Monk, pers. comm.) and that, in the seed stores of PGG Wrightson Seeds, mice rarely eat, nest in or damage endophyte-infected seed (A. Stewart, pers. comm.). The known effects of endophyte-infected grasses on mouse reproduction (Zavos *et al.*, 1987; Godfrey *et al.*, 1994) may further enhance any positive control of mouse populations induced by *Avanex*<sup>®</sup> which, in turn, has the potential to decrease the numbers of raptors. This could result in a decrease in raptor strikes which would be of great benefit to the aviation industry. In New Zealand, raptor species include the endemic New Zealand falcon (*Falco novaeseelandiae*), the native morepork owl (*Ninox novaeseelandiae*), the barn owl (*Tyto alba*) and the swamp harrier (*Circus approximans*). Raptors are also a recognized problem in the USA where they were responsible



**Figure 2** Percentage of E+ diet consumed by female (■) and male (△) mice over time in Trial 2. On day 14, the positions of the diet choices were reversed. Error bars represent standard error of the means; in some cases, error bars are too small to be visible.

for 13% of the total bird strikes where a species was identified in the time period 1990–2012. Furthermore, over the same time period, raptors were responsible for 20% of the bird strikes associated with damage (Dolbeer *et al.*, 2013) and their populations are known to increase rapidly at some US airports in certain seasons, some species by 200% (Caister, 2009).

Endophytes have also been shown to have effects on other mammalian species. For example, meadow voles (*Microtus pennsylvanicus*) are abundant in agricultural habitats and can cause significant damage to fruit trees, garden plants and commercial grain crops in North America. Planting endophyte-infected grasses has been shown to reduce vole damage to apple trees (Conover, 2003), and endophyte-infected diets can increase mortality at increased ambient temperatures (Conover, 1998). Related Prairie voles (*Microtus ochrogaster*) are negatively affected by endophyte-infected seed and showed suppressed food and water intake, growth rates and reproductive success compared to voles on an endophyte-free seed diet (Durham and Tannenbaum, 1998). Although this area of endophyte science is largely unresearched, many believe cool season grass endophytes are the way forward in many areas of wildlife management being more eco-friendly and sustainable than alternative less humane methods of control (Coley *et al.*, 1995). The use of Avanex® to control wildlife problems caused by other mammals could therefore also be explored as endophyte associations may deter feeding in other mammals as it has in mice.

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