Fate of biological control introductions: Monitoring an Australian fungal pathogen of grasshoppers in North America

(insect pathology/epizootiology/*Entomophaga grylli*/molecular probes)

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ABSTRACT   In North America there are two generally recognized pathotypes (pathotypes 1 and 2) of the fungus *Entomophaga grylli* which show host-preferential infection of grasshopper subfamilies. Pathotype 3, discovered in Australia, has a broader grasshopper host range and was considered to be a good biocontrol agent. Between 1989 and 1991 pathotype 3 was introduced at two field sites in North Dakota. Since resting spores are morphologically indistinguishable among pathotypes, we used pathotype-specific DNA probes to confirm pathotype identification in *E. grylli*-infected grasshoppers collected at the release sites in 1992, 1993, and 1994. In 1992, up to 23% of *E. grylli*-infected grasshoppers of the subfamilies Melanoplinae, Oedipodinae, and Gomphocerinae were infected by pathotype 3, with no infections >1 km from the release sites. In 1993, pathotype 3 infections declined to 1.7%. In 1994 grasshopper populations were low and no pathotype 3 infections were found. The frequency of pathotype 3 infection has declined to levels where its long-term survival in North America is questionable. Analyses of biocontrol releases are critical to evaluating the environmental risks associated with these ecological manipulations, and molecular probes are powerful tools for monitoring biocontrol releases.

Current widely publicized problems with synthetic chemical insecticides have given rise to a sense of urgency in the development of biological control agents as supplements or alternatives to these chemicals. Chemical control is not only often environmentally harsh but also at times ineffective; in 1987 chemical pesticides failed to control an outbreak of grasshoppers in the western United States. The application of non-native strains of entomopathogenic fungi offers tremendous potential in pest control but also demands a careful and responsible approach. There is a perception that non-native strains may behave very differently from native strains. One particular example that has aroused controversy is the release of an Australian pathotype of the entomopathogenic fungus *Entomophaga grylli* into the United States for the control of grasshopper populations (1–3). The perceived benefits of introducing foreign insect pathogens into the United States (3) has potential risks which include inhibition of natural pathogen effectiveness, possible threat to nontargeted organisms, loss of biological diversity, and disruption of food chains (1, 2).

There are three generally recognized pathotypes of *E. grylli*, all apparently specific pathogens of grasshoppers but with different grasshopper subfamily host ranges (4). The major pest grasshopper species in North America occur in the subfamilies Melanoplinae and Gomphocerinae, with localized outbreaks of Oedipodinae. Pathotypes 1 and 2 are endemic to North America and show preferential infection of different grasshopper subfamilies; under laboratory and field conditions pathotype 1 preferentially infects oedipodine grasshoppers whereas pathotype 2 infects melanopline grasshoppers. Pathotype 3, although isolated from a cyrtacanthacridine grasshopper in Australia (*Praxibulus* sp.), has a broader host range which includes North American oedipodine and melanopline grasshoppers under laboratory conditions (5). Its ability to infect North American gomphocerine grasshoppers was not known. Pathotype 3 was considered to be a good candidate as a grasshopper biological control agent because it held the potential to infect melanopline and oedipodine grasshoppers and, possibly, gomphocerine grasshoppers (3).

As part of a biological control trial to suppress rangeland grasshopper populations, pathotype 3 was introduced into McKenzie County, North Dakota, from 1989 to 1991 (6). The lack of basic information on the establishment, spread, host range, and epidemiology of the Australian pathotype (1, 2, 7) resulted in a decision to discontinue grasshopper control trials with exotic organisms (7). The inability to distinguish the Australian pathotype from endemic North American *E. grylli* pathotypes precluded a testable prediction of its effects on grasshopper populations and nontarget insects. Grasshoppers infected by *E. grylli* typically exhibit "summit disease syndrome," characterized by their clinging to elevated surfaces, such as stalks of wheat, prior to death (6). However, no morphological characters (resting spores, conidia, hyphal bodies) are adequate for separating the three pathotypes (8–10). In retrospect, we constructed cloned DNA fragments as pathotype-specific probes (11) to allow positive identification of the three *E. grylli* pathotypes at two release sites and surrounding areas during the three years (1992–1994) following the release of pathotype 3.

MATERIALS AND METHODS

Release of Pathotype 3-Infected Grasshoppers. Grasshoppers (*Melanoplus differentialis*) were injected with pathotype 3 protoplasts and released at two sites (A and B), ≈17 km apart, in McKenzie County, North Dakota, from 1989 to 1991 (12). Sites A and B are also known as Wold's field and Nelson's field no. 1, respectively. Site A was a mixed alfalfa stand whereas site B was primarily composed of crested wheatgrass. The number of pathotype 3-infected grasshoppers released each year at each site was as follows: site A, 500 grasshoppers in 1989, 2500 in 1990, 0 in 1991; site B, 0 grasshoppers in 1989 and 1990, 2000 in 1991. In the following years (1992, 1993, and 1994), dead grasshoppers that contained resting spores or conidia were collected near the release sites and areas concentric to the

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Population Biology: Bidochka *et al.*

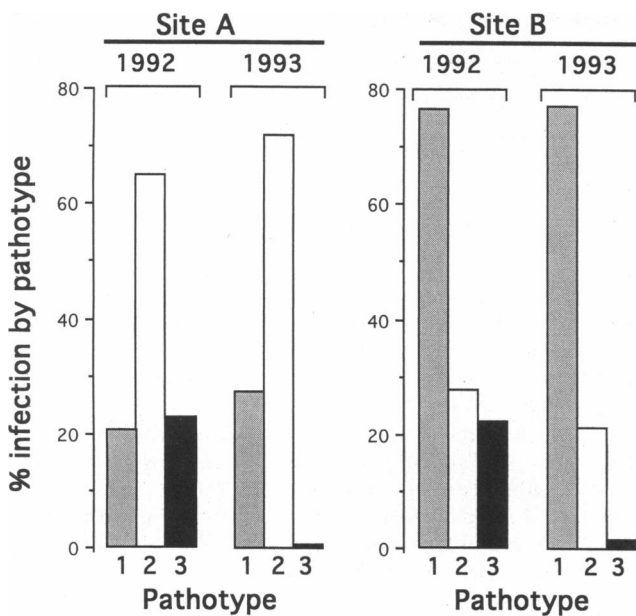*Proc. Natl. Acad. Sci. USA 93 (1996)*   919

FIG. 1. Frequency of pathotype 1, 2, or 3 infections, as determined by probe analysis, in *E. grylli*-infected grasshoppers collected within 1 km of sites A and B in 1992 and 1993. The sums may be greater than 100% due to coinfections. No pathotype 3 infections were found in 1994.

release sites (12) and analyzed for *E. grylli* infection by using the pathotype-specific probes.

Construction of Pathotype-Specific DNA Probes. The construction of *E. grylli* pathotype-specific cloned DNA probes has been reported (11).

Fracturing of Resting Spores and Extraction of DNA. To extract DNA from resting spores in dead grasshoppers the infected grasshopper was placed in 0.5 ml of 10 mM Tris/1 mM EDTA buffer (pH 7.5) in a 2.2-ml Eppendorf tube. Nymphal grasshoppers were homogenized whole, whereas the abdomens of adult grasshoppers were homogenized. Each sample was homogenized (Tissue Tearor, model 985-370, variable speed, setting 5, 30,000 rpm; Biospec Products, Inc.) on ice three times for 10 sec with a 5-sec interval between pulses.

DNA was extracted twice with an equal volume of phenol/chloroform and precipitated with 0.1 volume of 3 M sodium acetate and 1 volume of 2-propanol. The mixture was centrifuged and the pellet was washed with ice-cold 70% ethanol. The DNA pellet was resuspended in 100 μl of Tris/EDTA (pH 7.5) buffer. DNA was denatured with 40 μl of 1 M NaOH and incubated for 10 min at room temperature. Then 170 μl of 0.125 NaOH/0.125× standard saline citrate was added for a final volume of 310 μl. DNA (100 μl) was blotted onto nitrocellulose membranes in triplicate and each membrane was hybridized with a radiolabeled pathotype-specific DNA probe.

Bioassay of Pathotype 3 in Field-Collected Grasshoppers. Fifth-instar grasshoppers were collected for bioassay from McKenzie County, North Dakota, and surrounding areas in

1989–1991. Field-collected grasshoppers were housed in cages and fed wheat bran for 10 days prior to bioassay. Twenty-five grasshoppers in each of 12 species collected were injected with 10^4 protoplasts in 10 μl of Grace's insect tissue culture medium (12). Grasshoppers were checked daily for mortality. Dead grasshoppers were placed on water agar and checked for conidia formation within 24 hr of death. They were also dissected to determine whether resting spores were present in the body cavity.

RESULTS

Of the 1216 *E. grylli*-infected grasshoppers (888 within 1 km of sites A and B and 243 concentric to the release sites) that were collected in 1992–1994 and analyzed, 1127 (92.7% of total) showed positive signals for one of the three pathotypes. Fig. 1 shows the percent infection by each of the three *E. grylli* pathotypes, as determined by probe hybridization analysis, within 1 km of sites A and B in 1992 and 1993. Pathotype 3 had overwintered through to 1992 and in that year 23.0% (site A) and 22.5% (site B) of all *E. grylli*-infected grasshoppers were infected by pathotype 3. In 1993, pathotype 3 infection decreased ≈95% compared to 1992, with 0.6% (site A) and 1.7% (site B) infection.

In 1994 grasshopper populations were low and only 75 *E. grylli*-infected grasshoppers were collected, with 71 grasshoppers testing positive for pathotype 2. None of the grasshoppers collected in 1994 tested positive for pathotype 1 or 3. These data are not shown since the sample size was small.

Outside the 1-km radius from the release sites, 243 grasshoppers were collected (141 in 1992 and 102 in 1993) that gave positive signals (99.6% of total) by probe analysis for pathotype 1 or 2 but not for pathotype 3, indicating that pathotype 3 probably did not disperse from the release sites.

Table 1 shows the breakdown of single-pathotype infections by grasshopper subfamily and site in 1992 and 1993. Site A had a greater proportion of melanopline grasshoppers, while site B had more oedipodine grasshoppers. Frequency differences for pathotypes 1 and 2 at sites A and B (Fig. 1) reflect different species compositions and subfamily representations at the two sites (Table 1). Pathotype 1 preferentially infected oedipodine grasshoppers and pathotype 2 preferentially infected melanopline grasshoppers. However, pathotypes 1 and 2 showed 1.1–15.8% cross-infection from their preferred hosts, and pathotype 3 infected melanopline, oedipodine, and gomphocerine grasshoppers. In 1992, the frequency of pathotype 3 infection was highest in the Gomphocerinae at site A and in the Melanoplinae at site B.

Analysis of the dot blots revealed infection by more than one pathotype in individual grasshoppers (Table 2). In 1992, 9.0% (site A) and 15.5% (site B) of all *E. grylli*-infected grasshoppers showed coinfection of pathotype 3 with pathotype 1 or 2. The frequency of pathotype 3 with pathotype 1 or 2 coinfection was highest in the Gomphocerinae. Only one incident of a coinfection of pathotype 1 with pathotype 2 was observed, and that was in an oedipodine grasshopper at site B in 1992.

Table 1. Percent infection by *E. grylli* pathotypes at sites A and B in 1992 and 1993 by grasshopper subfamily

Grasshopper subfamily	Site A								Site B							
	1992				1993				1992				1993			
		Pathotype				Pathotype				Pathotype				Pathotype		
	n	1	2	3	*n*	1	2	3	*n*	1	2	3	*n*	1	2	3
Melanoplinae	153	2.0	78.3	12.5	113	3.5	95.6	0	39	15.8	57.9	18.4	54	5.7	94.3	0
Oedipodinae	44	65.9	11.4	11.4	36	91.7	8.3	0	139	74.1	2.9	5.0	179	96.6	1.1	2.3
Gomphocerinae	26	23.0	34.6	26.9	8	75.0	25.0	0	10	70.0	10.0	0	12	100.0	0	0

Values in 1992 may not equal 100%, due to coinfections (see Table 2). *n* is the sample size.

Table 2. Percent coinfection by *E. grylli* pathotypes (1 + 2, 1 + 3, or 2 + 3) at sites A and B in 1992 by grasshopper subfamily

Grasshopper subfamily	Site A			Site B		
	1 + 2	1 + 3	2 + 3	1 + 2	1 + 3	2 + 3
Melanoplinae	0	0.7	6.5	0	2.6	5.3
Oedipodinae	0	9.1	2.3	0.7	17.3	0
Gomphocerinae	0	11.5	3.9	0	20.0	0

No coinfections were observed in 1993. Sample sizes are as in Table 1.

Combining the single-pathotype infections with the coinfections, pathotype 3 infections were represented equally well (19.6–22.3%) in all three grasshopper subfamilies in 1992. The exception was that 42.3% (26.9% pathotype 3 infection plus 11.5% and 3.9% pathotype 3 coinfections) of the gomphocerine grasshoppers at site A in 1992 were infected by pathotype 3. However, the relative number of *E. grylli*-infected gomphocerine grasshoppers was low.

Table 3 shows the occurrence of *E. grylli* pathotypes in the infected grasshopper species collected at sites A and B in 1992 and 1993. The three most common *E. grylli*-infected grasshopper species were *Melanoplus sanguinipes*, *Melanoplus bivittatus*, and *Camnula pellucida*. This table also shows host preferences by the pathotypes in grasshopper species. Pathotype 2 infected the oedipodine grasshopper *Metator pardalinus*; however, only two of these grasshoppers were collected. All other grasshopper species were preferentially infected by their respective pathotypes, although higher levels of pathotype 3 infections were noted in *Melanoplus femurrubrum* and *Ageneotettix deorum* in certain instances. We have also shown that, along with melanopline grasshoppers, gomphocerine grasshoppers were preferentially infected by pathotype 1. No *E. grylli*-infected *Hesperotettix viridis* were found at the field release sites, and only one *H. viridis* was collected at these sites between 1989 and 1994. Sites A and B were without snakeweed, a food plant for *H. viridis*. For laboratory bioassay, *H. viridis* was collected at a field that contained snakeweed.

Table 4 shows the results of the laboratory bioassays of pathotype 3 in field-collected grasshoppers. Pathotype 3 caused mycosis in melanopline, oedipodine, and gomphocerine grasshoppers under laboratory conditions. The grasshop-

per species host range of pathotype 3 in laboratory bioassays was broader than that of infection of grasshopper species by this pathotype found in the field. Laboratory infections by pathotype 3 were observed in *M. differentialis*, *M. confusus*, and *Aeropedellus clavatus*, but field infections by pathotype 3 in these grasshoppers were not found. *Phoetaliotes nebrascensis*, *Spharagemon collare*, *M. pardalinus*, and *Chorthippus curtipennis* were not represented in the laboratory bioassay and pathotype 3-infected cadavers were not found in the field. *H. viridis* was not infected by pathotype 3 in the laboratory bioassay, nor were pathotype 3-infected cadavers found at the two field sites.

DISCUSSION

We have utilized pathotype-specific probes for the entomopathogenic fungus *E. grylli* (11) in order to monitor the establishment of an Australian pathotype 3 introduced into North Dakota between 1989 and 1991. Our results suggest that pathotype 3 caused substantial infection ($\approx$23% of *E. grylli*-infected grasshoppers) in 1992, but in 1993 infection declined to <2% and no pathotype 3 infections were found in 1994. Molecular markers have previously been used to identify an exotic entomopathogenic fungus in North America (13). *Entomophaga maimaiga*, an Asian fungus, was identified as the causative agent for gypsy moth epizootics in eastern North America, and the geographical range of this fungus is increasing (13). These studies show that molecular probes are powerful tools for epizootiological studies involving biocontrol agents.

Table 3. Infection occurrence of *E. grylli* pathotypes in grasshopper species and frequency of *E. grylli*-infected grasshoppers at sites A and B in 1992 or 1993

Grasshopper subfamily	Species	Pathotype*			Grasshopper frequency†
		1	2	3	
Melanoplinae	*Melanoplus sanguinipes*	x	O	x	H
	Melanoplus bivittatus	x	O	x	H
	Melanoplus packardii	x	O	x	M
	Melanoplus infantilis	x	O	x	L
	Melanoplus gladstoni	x	O		L
	Melanoplus femurrubrum‡		O	O	L
	Melanoplus differentialis		O		L
	Melanoplus confusus		O		L
	Phoetaliotes nebrascensis		O		L
Oedipodinae	*Camnula pellucida*	O	x	x	H
	Spharagemon collare	O			L
	Metator pardalinus		O		L
Gomphocerinae	*Ageneotettix deorum*§	O	x	O	M
	Aulocara ellioti	O	x	x	M
	Aeropedellus clavatus	O			L
	Chorthippus curtipennis	O			L

*x indicates the occurrence of a pathotype infection in a grasshopper species. O indicates that >60% of the individuals in the species were infected by that pathotype in 1992 or 1993.
†A measure of *E. grylli*-infected grasshopper frequency; H, high (>100 *E. grylli*-infected grasshoppers collected); M, medium (<100 but >20); L, low (<20).
‡Sites A and B, 1992, >60% pathotype 3 but at site B, 1993, >60% pathotype 2.
§Site A, 1992, >60% pathotype 3 but at site B, >60% pathotype 1.

Population Biology: Bidochka *et al.*

Proc. Natl. Acad. Sci. USA 93 (1996) 921

Table 4. Results of laboratory bioassay of pathotype 3 in field-collected grasshoppers

Grasshopper subfamily	Species	Pathotype 3 infection
Melanoplinae	*Melanoplus sanguinipes*	+
	Melanoplus bivittatus	+
	Melanoplus packardii	+
	Melanoplus infantilis	+
	Melanoplus femurrubrum	+
	Melanoplus differentialis	+
	Melanoplus confusus	+
	Phoetaliotes nebrascensis	+
	Hesperotettix viridis	−
Oedipodinae	*Camnula pellucida*	+
Gomphocerinae	*Ageneotettix deorum*	+
	Aulocara ellioti	+

Twenty-five fifth-instar grasshoppers were bioassayed for each species. Assessment of infection: +, >70% of grasshoppers died from pathotype 3 infection 5–10 days after injection of protoplasts; −, <10% of grasshoppers died but no mycosis was found.

The ecological principles implicated in the possible failure of pathotype 3 to establish in North. America have been outlined by Lockwood (1); these include competitive exclusion, invasion ecology, and community resistance to perturbation. The differential susceptibility among grasshopper species to *E. grylli* infections and any changes in the species mixes may affect the frequency of occurrence of pathotype 3. Grasshopper species densities and the phenology of occurrence, in addition to their ratios, could play a role in pathotype-specific infections of grasshoppers from the field. Migrant grasshoppers infected by pathotype 1 or 2 would further dilute the effects of pathotype 3. Despite optimistic arguments for the establishment of exotic biological control agents of grasshoppers in North America (3), the relative frequency of pathotype 3 has declined to levels so low that its maintenance in North Dakota may be difficult without more releases. Even if pathotype 3 resting spores are able to persist in soils and through winters in North Dakota, its future impact on suppression of grasshopper populations to a greater extent than pathotypes 1 and 2 would probably be minimal since biological control that is based on a rare species is likely to be ineffective (14).

Coinfections of pathotype 1 or 2 with pathotype 3 were observed. The technique we employed cannot distinguish between an infection by a recombinant strain from an infection derived by two separate pathotypes. The formation of stable recombinant strains could have an adverse impact on the viability and continuation of native pathotypes, the loss of which could severely affect the prairie ecosystem (1, 2). However, *E. grylli* resting spores are formed by azygosporogenesis (15) with no apparent sexual recombination, precluding the possibility of recombinant strains. Pathotypes 1 and 2 appear to be separated with respect to host coinfection since there was only one incident of coinfection by these pathotypes. Host-specific cuticular cues, phenology of infection, and competitive exclusion are some of the factors that may be responsible for separating pathotype 1 from pathotype 2 in host grasshoppers. As expected, pathotypes 1 and 2 showed host preferences for oedipodine and melanopline grasshoppers, respectively, but host switching was detected in nature. We also showed that, in the field, gomphocerine grasshoppers were preferentially infected by pathotype 1.

Lockwood (1, 2) expressed concern that pathotype 3 may infect "beneficial" grasshoppers or suppress certain grasshopper species, resulting in the disruption of the local prairie ecosystem. *M. femurrubrum* is an intermediate host of a parasite of several native bird species, and suppression of this grasshopper species on a large scale could have ecological

impacts (1). In 1992, half of the *E. grylli*-infected *M. femurrubrum* were infected by pathotype 3. This could represent some ground for concern if large populations of this grasshopper were present. However, only 12 *E. grylli*-infected *M. femurrubrum* were collected. No *E. grylli*-infected *H. viridis* were found in the field. This grasshopper is viewed as a beneficial species because it feeds on a noxious weed, snakeweed (1, 7). Snakeweed was not common at either site and the *H. viridis* that were bioassayed in the laboratory were collected ≈10 km from the field release sites. Under laboratory conditions, this grasshopper was the only species tested that was not infected by pathotype 3. The host range of pathotype 3 was broader in the laboratory (physiological host range) than in the field (ecological host range). Physiological and ecological host ranges of entomopathogenic fungi may differ, and caution should be exercised when extrapolating laboratory bioassay results to potential infections in the field.

In light of the controversy surrounding the introduction of the exotic strain, the failure of pathotype 3 to establish in 1993 and 1994 after a relatively high infection in 1992 is one of the best possible outcomes in this biological control trial. Persistence of pathotype 3 could result in ecological disruption of the prairie community (1, 2). However, previous to its decline, epizootics caused by pathotype 3 in 1992 may be, in part, responsible for the recent decrease in grasshopper populations (16), and in 1994 only 75 *E. grylli*-infected grasshoppers were collected near the sampling sites. Pathotype 3 may have helped to cause a reduction in the local grasshopper populations in 1992 and thus eliminate susceptible hosts. Mainly targeted, economically important grasshoppers were affected and no catastrophic effects on the natural community have been noted.

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