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ASSESSMENT OF HEALTH AND GROWTH OF RING-NECKED PHEASANTS FOLLOWING CONSUMPTION OF INFECTED INSECTS OR CONIDIA OF ENTOMOPATHOGENIC FUNGI, Metarhizium anisopliae var. acridum AND Beauveria bassiana , FROM MADAGASCAR AND NORTH AMERICA

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ASSESSMENT OF HEALTH AND GROWTH OF RING-NECKED PHEASANTS FOLLOWING CONSUMPTION OF INFECTED INSECTS OR CONIDIA OF ENTOMOPATHOGENIC FUNGI, *Metarhizium anisopliae* var. *acridum* AND *Beauveria bassiana*, FROM MADAGASCAR AND NORTH AMERICA

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*Isolates of two fungi (*Beauveria bassiana *and* Metarhizium anisopliae *var.* acridum*) from Madagascar are being developed for control of grasshoppers and locusts, as part of a search for alternatives to environmentally detrimental chemical insecticides. The probable effects of these entomopathogens on nontarget species must be determined before operational use. Birds may become exposed to these fungi either directly, by consuming spores deposited on their food items, or secondarily, by consuming grasshoppers or locusts that have died from infection by these biocontrol agents. This article presents the results of per os challenge from fungus-infected food items. Male and female ring-necked pheasants (*Phasianus colchicus*) were exposed at 4 d of age and again at 9 d of age to challenge treatments, or 2 control treatments (18 male and 18 female birds per treatment group). Pheasants were weighed at 9, 17, and 25 d of age, tarsal length was measured at 25 d of age, and they were observed daily for signs of adverse effects of the experimental treatments. At the time of euthanasia (25 d of age), 2 or 3 randomly selected birds from each of the groups exposed to infected grasshoppers, plus the 2 control groups, underwent*

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complete necropsies and histopathological examination of 16 tissues from each bird. Results show that in both sexes, weight gain at both 17 and 25 d was not significantly different between challenge groups and control groups. Tarsal length at 25 d of age, an indication of structural growth, was also not markedly different among challenged and control groups. Histopathological changes were generally undetectable, mild, or moderate, and not consistently associated with any treatment. Based on these findings, there is little indication that birds are susceptible to detrimental health effects from direct or secondary exposure to the two entomopathogenic fungi studied.

Grasshoppers and locusts (Orthoptera: Acrididae) are often serious agricultural pests in grassland biomes, such as Sahelian Africa (Prior et al., 1992) and the plains of North and South America. The outbreak of desert locust (*Schistocerca gregaria*) and related acridids in Africa in the late 1980s affected 23 nations, and required over U.S. $250 million in donor aid for control efforts and alleviation of damage caused by locusts (Showler & Potter, 1991). Large areas on the Canadian Prairies are infested in some years. Between 1985 and 1991, grasshopper infestations necessitated spraying of 7.7 million hectares in Saskatchewan and Alberta, with an economic loss of at least $326 million CAN (Johnson et al., 1996).

Impacts of chemical insecticides on avian wildlife as a result of control actions during outbreaks of acridids have been reported. Suspected impacts of toxicity to burrowing owls (*Speotyto cunicularia*) (James & Fox, 1987) and other birds (Leighton et al., 1987) resulted in the deregistration of carbofuran for grasshopper control in Canada (AAFC, 1993; PMRA, 1995). The grasshopper insecticide monocrotophos accounted for over 4000 deaths of Swainson's hawks (*Buteo swainsoni*) in Argentina during a single growing season (Goldstein et al., 1999). In Africa, insecticides used for grasshopper and locust control have potential direct and indirect impacts on grassland birds (Mullie & Keith, 1993).

As part of a worldwide search for alternatives to environmentally harsh chemical insecticides used to control these pests, isolates of the fungi *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* var. *acridum* Driver & Milner are being developed for control of grasshoppers and locusts (reviewed by Jaronski & Goettel, 1997; Lomer et al., 1997, 2001; Milner, 1997). Recent field experiments in Madagascar with indigenous isolates of *M. anisopliae* var. *acridum* resulted in over 70% mortality of grasshoppers and locusts [*Locusta migratoria capito* (Sauss)] 10–12 d after treatment (Swanson, 1997). The feasibility of production of mycoinsecticide in Madagascar for this purpose has been investigated (Swanson, 1997). Modest levels of control of grasshoppers has been observed in other field studies (Johnson & Goettel, 1993). Performance resulting in less than complete grasshopper mortality represents a reasonable target efficacy, because such treatments are capable of providing adequate crop protection while leaving a food resource sufficient for survival and reproduction of grassland songbirds (Martin et al., 1998, 2000).

Beauveria bassiana isolates with activity against grasshoppers and locusts have been obtained from insect and soil samples in Montana (Mycotech,

Corp., Butte, MT), Alberta (Research Centre, Lethbridge, AB, Canada), and Africa (Benin, Paraïso et al., 1992; Burkina Faso, Ouedraogo, 1993). In Canada and the United States, research and development of isolates of this fungus have been in progress since 1987 (Marcandier & Khachatourians, 1987; Moore & Erlandson, 1988). Conidia from Mycotech isolate "GHA" (isolated from a chrysomelid from Oregon and subsequently passed through the grasshopper *Melanoplus sanguinipes* before fermentation production) were field tested in aqueous and powdered formulations in Alberta (Johnson et al., 1988) and were applied in oil formulation against African grasshoppers (Johnson et al., 1992). Field reductions in grasshopper population density of 60% were obtained after application of dry *B. bassiana* conidia under cool and overcast conditions (Johnson & Goettel, 1993), but under warmer environmental conditions such as is typical of the Sahel of West Africa, effectiveness is reduced (Johnson et al., 1992). Inglis et al. (1996, 1997a, 1997b) measured a cardinal temperature of 33°C for growth of *B. bassiana. Metarhizium anisopliae* var. *acridum* has been shown to be active at higher temperatures (Inglis et al., 1996; Thomas & Jenkins, 1997). This effect may allow *M. anisopliae* var. *acridum* to be more effective in sunny conditions that allow basking by the target insects (depending on intensity and direct effects; Blanford et al., 1998; Moore et al., 1996; Welling et al., 1995), and subsequent elevation of body temperature to greater than 35°C. Grasshoppers will adjust their positions to attain a preferred body temperature of 38–39°C (Lactin & Johnson, 1996a, 1996b, 1998).

The activity of *M. anisopliae* var. *acridum* at higher temperatures may enhance mortality of infected grasshoppers and locusts in warm seasons, but may represent a potential threat to nontarget, warm-blooded species. Critical issues of toxicity and host specificity must be addressed for health, environmental safety, and regulatory concerns (Prior, 1990). Experimental studies with *B. bassiana* indicate the potential for damage to nontarget insects, such as predaceous beetles (Magalhaes et al., 1988; Pingal & Lewis, 1996). *Metarhizium anisopliae* var. *acridum*, like *B. bassiana,* has shown some activity against honeybees, but this effect varies considerably among fungal isolates (Ball et al., 1994).

Field observations by LUBILOSA (Lutte Biologique contre les Locusts et les Sauteriaux; Lomer, 1996) indicate that predation rates on fungus-infected grasshoppers and locusts could be higher than on healthy insects of the same species. Apart from the likelihood of enhanced biological control through successful predation, this observation further emphasizes the importance of studies simulating the consumption of diseased insects by birds. Young precocial birds may be at greater risk if they feed more heavily on weakened insects, whereas altricial nestlings may have higher exposure when parent birds collect the infected, moribund insects for their offspring.

The mycotoxin oosporein, isolated from *B. bassiana* (Vining et al., 1962), is toxic to birds (Cole et al., 1974; Pegram & Wyatt, 1981; Pegram et al., 1982; Manning & Wyatt, 1984), yet not all isolates of this fungus produce this metabolite, and most *B. bassiana* isolates will not grow at avian

body temperatures, which generally exceed 38°C. Althouse et al. (1997) found that American kestrels that ingested spores of one isolate of *B. bassiana* at 5×10^6 spores/g body weight (simulating encounter under typical field application rates) developed no gross pathological lesions or alterations of behavior. Viable spores have been reported from avian feces (Hartmann & Wasti, 1980), indicating a need for histological examination of exposed birds.

Use of an insectivorous bird for avian testing is a requirement of the Microbial Pesticide Test guidelines (OPPTS 885.4050, Avian Oral, Tier 1) of the U.S. Environmental Protection Agency. The ring-necked pheasant (*Phasianus colchicus*) was chosen as a model that would reflect potential impacts of *Beauveria* and *Metarhizium* mycoinsecticides on wild birds in Africa or Canada. This species has been identified as both a valuable representative species (a species that represents a group of species too numerous to study individually) and a surrogate species (a substitute for a species of concern that is itself unavailable for direct study) under the definitions of the Avian Effects Dialogue Group (Rymph, 1994). Other useful features of the ring-necked pheasant as a test species are (1) a natural readiness to feed on insects including grasshoppers (Whitmore et al., 1986); (2) anatomical, ecological, and physiological similarities to other upland game birds; (3) the large amount of comparative data that has been generated through its use as a common test species for chemical insecticides; and (4) the cosmopolitan biogeography and ecology of galliforms, including the Phasianidae. The ring-necked pheasant is firmly established over much of North America, where it shares its range in the Canadian Prairies and the interior grasslands of British Columbia with sharp-tailed grouse (*Tympanuchus phasianellus*) and gray partridge (*Perdix perdix*), game birds whose natural diets in include grasshoppers as a significant component (Jones, 1966; Johnsgard, 1973; Weigand, 1980). Grasshoppers are also known to be important food items for juvenile sage grouse (*Centrocercus urophasianus*), an endangered species in Canada (Johnson & Boyce, 1990; Klebenow & Gray, 1968).

The purpose of our study was to assess the potential for negative effects on the health of birds consuming mycoinsecticide-infected grasshoppers and locusts. The principal objectives were (1) to determine whether the presence of the entomopathogenic fungi in the food items had a measurable impact on the appetite, growth, development, general behavior, survival, or health of the birds, (2) whether these fungi could establish infections, and (3) whether and for how long the fungi could be detected in the feces and tissues of treated birds.

MATERIALS AND METHODS

Avian Test Species

Uniform, day-old, ring-necked pheasant chicks were obtained from mass-reared broods at the Brooks Wildlife Center (Alberta Forestry, Lands and Wildlife, Brooks, AB, Canada), using the same methods and source of birds

used in previous and subsequent avian toxicity tests with grasshopper insecticides (Martin et al., 1996; Smits et al., 1999). Chicks were maintained in the Controlled Environment Barn, Agriculture Canada Research Facility, Lethbridge, AB, and fed unmedicated duckling starter (21% duck and goose starter crumbles, Unifeed, Lethbridge, AB—21% protein, 3% crude fat, 5% crude fiber, supplemented with vitamins A, D, E) until being exposed to the challenge diets.

Experimental Design

Each replication (i.e., complete set of the treatments) included 60 birds, with 6 birds (3 of each sex) in each of 10 treatment groups. The experiment was replicated 6 times, resulting in 18 males and 18 females per treatment or a total of 360 birds. Treatments were assigned randomly to birds and pens within each replication. Birds and cages were labeled with code numbers in random order so that pathology observations were conducted "blind" with regard to treatment identities. Each experimental group was housed in a newly constructed wooden pen (1.7 m × 1.2 m × 0.6 m high) equipped with two 74-W brooder lamps, water, feed trays, and wood-chip bedding over black plastic sheets. The birds were confined and observed in the pens for 16 d after treatment.

Experimental Treatments

Birds receiving grasshopper diet treatments (infected or uninfected) were first isolated for 5–10 min. Each bird was then fed two grasshoppers (live, per capita body weight of fourth-instar nymphs was 0.21–0.25 g), on each of 2 d separated by 4 d without treatment (June 6 and 11). At the age the pheasants were being treated (4 and 9 d old), two grasshoppers were equivalent to approximately 2% of the body weight of the birds. The feeding rate of two grasshoppers per chick per feeding event is in line with the number of grasshoppers found in the diets of chicks of other grassland birds species (Martin et al., 1998, 2000), and approximately matches the dosing methods used in previous studies (Martin et al., 1996). In field applications (e.g., Johnson & Goettel, 1993, and others summarized by Lomer et al., 2001), complete or near complete infection of grasshoppers has not been observed, although complete or near complete infection of treated grasshoppers is typical in cage tests. Under field conditions, a proportion of grasshoppers slowly show evidence of infection, and may or may not die. Typically infection of a less intense level than in this study is found in about half of the population of grasshoppers in the treated areas. Birds feeding on the treated population would be expected to obtain a mix of infected and uninfected grasshoppers and other insects (and vegetation, depending on the species and age of bird). In our study, all of the grasshoppers used in the fungal treatments showed obvious signs of infection.

The fungal isolates of *Metarhizium anisopliae* var. *acridum* used in the challenge diets were supplied by Mycotech Corp. (Butte, MT) and Montana

State University (Bozeman, MT), and originated from *Locusta migratoria capito* (Sauss) in Madagascar (ARSEF number 5734, "SP3"; ARSEF number 5736, "SP9"). The *Beauveria bassiana* used in the study was isolate GHA (= isolate number 726) provided by Mycotech Corp. The 6 fungal treatments were MSP3-1, MSP3-2, MSP9-1, MSP9-2, and GHA-1 and GHA-2, where "1" indicates that the food items were grasshoppers that died from infection, and "2" indicates grasshoppers that died from infection and were held for 3 d in high humidity to encourage maximum infection and possible sporulation. The other four treatments included two groups receiving *M. anisopliae* var. *acridum* (MSP9) spores on duckling starter diet (FC, fungal-coated) and two untreated control groups per sex, one of which was fed healthy uninfected grasshoppers (control-1), and a control group that received untreated duckling starter diet (control-2). Although this experiment primarily concerned assessment of the impact on birds of consumption of infected insects, the spore-coated treatments were included to simulate a product spill, and possibly indicate whether further research on this mode of challenge would be of interest. Before and after grasshopper feeding, all birds were maintained on a diet of duckling starter uncontaminated with fungal treatments.

Preparation of Infected Insects and Diets

Third and fourth-instar nymphs of the migratory grasshopper, *Melanoplus sanguinipes* Fabr., and second-instar nymphs of the Madagascar migratory locust, *Locusta migratoria capito* (Sauss), were determined in preliminary tests to be readily accepted prey items of young ring-necked pheasants, whether the insects were alive or freshly killed, infected or uninfected. The insects used for the main experiment were fourth-instar *M. sanguinipes* hatched from eggs laid by field-collected adult grasshoppers in cages at the Lethbridge Research Centre, and reared on a diet of wheat leaves, lettuce, and wheat bran. Before being fed to the birds, the grasshopper nymphs were infected with one of the three fungal isolates, using methods that simulate direct and secondary contact between grasshoppers and sprayed vegetation in the field. Dry spores of the three fungal isolates were formulated in water [with the wetting agent Silwet L-77 (Loveland Industries, Greely, CO) recommended by Mycotech] and applied to groups of grasshoppers and their wheat-leaf food in glass cages using a hand-held mist sprayer calibrated before and after application. The viability of the spores used in the treatments was determined by germination on PDA agar with benlate at 24 h at room temperature. The estimates were as follows: *Beauveria bassiana* 726, 83%; *Metarhizium anisopliae* var. *acridum* SP3, 70%; *Metarhizium anisopliae* var. *acridum* SP9, 54%. These estimates of germination rate were used to adjust the concentrations of spores in the treatments. The resulting dose was $1–1.5 \times 10^5$ viable conidia per insect. In addition, lettuce-leaf wafers (0.7 cm diameter) were treated with conidia suspended in sunflower oil (2-μl aliquots containing 10^5 conidia per wafer) and confined with grasshoppers in glass vials for 24 h. Viability, dispersion, and concentration of conidia

were determined with a hemocytometer and microscopic examination. Within 5–8 d, the infected grasshoppers typically exhibited symptoms ranging from slight lethargy, to discoloration and death. Subsequent treatment of lettuce wafers was conducted at the same rate, scaled up to allow application with a fine mist sprayer. The rates used were selected to simulate the approximate rates that would be encountered in effective operational-scale treatment of grasshoppers and locusts (Lomer et al., 1997, 2001).

The diet contaminated with spores *M. anisopliae* var. *acridum* was prepared by mixing duckling starter with dry spores of the fungi. Duckling starter (density 0.62 g/ml; particle mean mass = 0.35 g, typical range 0.27–0.45 g) was mixed with spores at a rate of 0.4 g conidia powder per 100 ml feed. Conidia powder concentration varied with batch and species (*M. anisopliae* var. *acridum* 8.4×10^9 conidia/g), so that the final concentration of spores was 5.3 to 6.4×10^7 conidia/g of dry duckling starter. This high concentration of spores simulated a bird feeding in the area of a product spill.

Growth and Survival

Individual birds were weighed at 4, 9, 17, and 25 d of age. Individual change in weight was determined for each bird from 9 to 17, 17 to 25, and 9 to 25 d of age. Tarsus length was measured at 25 d of age using dial readout calipers (tarsal length did not vary appreciably in the day-old chicks). Chicks were observed daily for any clinical signs of physiological or pathological effects associated with treatment. Clinical signs to be recorded included unusual vocalization or blinking, change in gait or coordination, other neurological signs, respiratory distress, anorexia, postural changes, ruffled or denuding of feathers, prolonged sternal recumbency, weakness, or decreased responsiveness to investigator presence. Any evidence of gastrointestinal disorder (i.e., vomition or diarrhea), nasal or ocular discharge, cyanosis of skin or mucous membranes, skin lesions, emaciation, or mortality was recorded.

Sixteen days after completion of the dietary exposure, samples of feces and blood were tested for the presence of *B. bassiana* or *M. anisopliae* var. *acridum,* by plating smears on three selective media: (1) oatmeal agar, Dodine, crystal violet, streptomycin, penicillin (Beilharz et al., 1982); (2) agar, bile, glucose, peptone, rose bengal, Dodine, chloramphenicol, cyclohexamide (Veen & Ferron, 1966); and (3) Sabouraud dextrose agar, benomyl, copper sulfate. (Of these, only the second is confirmed as demonstrably selective for *Metarhizium.*) Fresh stained and unstained samples of feces and blood were examined for signs of hyphael growth using Nomarski differential interference contrast microscopy.

Weight gain and growth measurements were compared among treatments with repeated measures (mixed model) analysis of variance, and analysis of variance of tarsal measurements and weight gain (randomized complete block design). Comparisons of means were made using Tukey's HSD

and Scheffé's test (SAS Institute, Inc., 1990). The criterion of significance for the tests of comparisons of means was set at $\alpha = .05$.

Observers watched for unusual vocalization or blinking, change in gait or coordination, respiratory distress, anorexia, postural changes, ruffled or denuding of feathers, prolonged sternal recumbency, weakness, decreased responsiveness to investigator presence, or other neurological symptoms. Observers also watched for and recorded any evidence of gastrointestinal disorder, nasal or ocular discharge, cyanosis of skin or mucous membranes, skin lesions, or emaciation.

Histopathology

A small proportion (two birds from each treatment group) were selected at random for histological analysis. They were euthanized by CO_2 inhalation. The following organ and tissue samples were collected within 60 min of death: brain, thymus, heart, lung, crop, proventriculus, ventriculus, duodenum, jejunum, cecum, colon, pancreas, spleen, liver, kidneys, and bursa of Fabricius. Tissues were fixed in 10% neutral buffered formalin and transferred to the Toxicology Centre (Saskatoon, SK), where they were embedded in paraffin blocks, sectioned at 5 μm, and stained with hematoxylin and eosin, or Gomori's methenamine silver nitrate, for histological examination. Tissues were examined for changes that could have resulted from mycotoxin damage or mycotic infection.

Deviations from normal, such as inflammation, necrosis, alterations to normal cellular structure, disruption of tissue architecture, or evidence of invasion by infectious agents, were assessed. Each tissue was evaluated and ranked according to predetermined categories for that organ. All tissues were ultimately scored using a numbering system of 1 to 4. Tissues with no abnormal findings, or mild artifactual tissue changes, were rated as "1." Tissues showing areas with mild pathological changes (lesions), or up to two small foci of more distinct pathology, were rated "2." A score of "3" indicated that there were numerous mild, or at least one large lesion, and any tissue with severe or extensive pathological change was rated "4." All the birds and tissues were labeled using identification codes so the pathologist was unaware of the treatment received by the individual animals.

Quantification of the Spores on Feed Treatments

Pheasant weights at ages 4, 9, 10, 16 (actually weighed at 16–17 d), and 46 d were used to fit a mass predictive equation. Mean masses were 26, 75, 87, 124, and 305 g, respectively ($n = 50, 358, 50, 358, 35$). The 10-d-old and 46-d-old birds were not part of the same group obtained for this experiment, but their weights were approximated by the same linear model. Sexes were approximately similar in weight during this age period, and equal numbers of the two sexes were weighed. Mass (g) as a function of age (d) during this period is approximately linear. Least squares regression equation (SAS Institute, Inc., 1990) yielded the simple linear equation

$$\text{Mass} = 14.90 + 6.38 \times \text{age} \quad (r^2 = .99)$$

During this period, the pheasants consume feed in proportion to body weight, and total feed requirements per pheasant were approximately 1000 g for 42 d. The solution for the integral of the linear equation provides the approximate consumption from the beginning of exposure (at 4 d to 16 d):

$$[14.9 \times 16 + (16^2/2) \times 6.38] - [14.9 \times 4 + (4^2/2) \times 6.38] = 944.4$$

Relative to the total for 42 d, this represents

$$944.4/[14.9 \times 42 + (42^2/2) \times 6.38] = 0.151 \times 1000 \text{ g} = 151 \text{ g}$$

Therefore, 151 g is an approximate estimate of the amount eaten during the age 4 to 16 d, during which time average pheasant mass increased from a mean of 40.4 to a mean of 117 g.

Wasted (unconsumed) food was estimated to be approximately 10% of the feed provided in trays. Total spore consumption during 12 d of treatment was approximately 151 (0.9) (5.4×10^7) = 7.4×10^{10}. If pheasants were consuming 0.16 g feed/g body weight/d, averaged over the integrated treatment period, then the daily dose was 8.6×10^6 spores (MSP9).

RESULTS

Ten birds died and were replaced during the initial stages of shipment and handling during experimental setup, prior to treatment. During the remainder of the 15-d experiment, only 2 birds died (leaving $n = 358$). One was a female in the control group, which received uninfected grasshoppers, in block 1; the other mortality was a male in the MSP9 treatment of block 4. There was no reduced survival among birds exposed to the diets that contained fungal treatments within the period of the test.

The birds readily consumed the infected insects during the period of exposure to the experimental diets, although in some cases the sporulated grasshoppers had to be placed in a bird's mouth via forceps before they would consume them. During the 16-d observation period surrounding treatment, there was no evidence of differences in behavior among the treatment groups, nor were there any cases of birds that showed clinical evidence of infection or disease.

Mean male body weight across all treatments at 9, 17, and 25 d of age was 25.9 g (SD 2.9), 74.8 g (SD 9.6), and 124.3 g (SD 18.3), respectively ($n = 179$). Treatment-specific data are in Table 1A. There was no evidence that consumption of the infected grasshoppers affected weight gain of the male pheasants (repeated measures mixed model ANOVA, $p = 0.065$; Table 2).

Mean female weight at corresponding ages was 27.4 g (SD 4.7), 75.6 g (SD 11.4), and 130.7 g (SD 20.4) ($n = 179$). The detailed data for females are in Table 1B. No significant differences occurred among the mean weight

TABLE 1A. Comparison of Body Weight Gain (g) and Structural Growth (Tarsal Length, mm) Among Young Pheasants from 9 to 25 d of Age on 10 Different Dietary Treatments Based on Fungal-Infected Grasshopper Food Items, Fungal-Coated (FC) Duckling Starter, Untreated Grasshoppers, or Untreated Duckling Starter

Dietary treatment	*n*	Sex	Weight gain for 9–17 d of age (0–8 d after treatment)[a] (g)	Weight gain for 17–25 d of age (8–16 d after treatment)[a] (g)	Weight gain for 9–25 d of age (0–16 d after treatment)[a] (g)	Tarsus 25 d of age (16 d after treatment)[a] (mm)
A. Male						
Control-1 Untreated grasshoppers	18	M	48.62	54.13	102.74	47.28
Control-2 Untreated duckling starter	18	M	53.17	55.84	109.01	48.17
Feed-1 FC duckling starter	18	M	47.15	50.18	97.33	46.56
Feed-2 FC duckling starter	18	M	48.06	52.39	100.45	47.54
Isolate MSP3-1[b] *M. anisopliae* var. *acridum*	18	M	46.69	54.79	101.49	47.94
Isolate MSP3-2[c] *M. anisopliae* var. *acridum*	18	M	48.21	58.07	106.28	48.35
Isolate MSP9-1[b] *M. anisopliae* var. *acridum*	18	M	46.68	56.81	103.49	48.94
Isolate MSP9-2[c] *M. anisopliae* var. *acridum*	17	M	44.74	50.26	95	47.04
Isolate GHA-1[b] *B. bassiana*	18	M	49.15	53.58	102.73	47.92
Isolate GHA-2[c] *B. bassiana*	18	M	53.04	61.29	114.33	48.69
Tukey's HSD minimum significant difference (df = 119)			10.43	14.06	20.54	4.2
Scheffé's test minimum significant difference (df = 119)			13.59	18.31	26.75	5.47

gains for males for any period (Table 2). A weak overall significant difference was observed for females (repeated measures mixed model ANOVA, $p = 0.005$). The weight gains of one of the groups of MSP3-treated birds were significantly lower (Table 1B). The weight gains of birds that had eaten MSP9-infected grasshoppers, with either acute or advanced sporulating infections, did not differ from the weight gains of any of the four control groups, with no evidence of pathological impact.

Histopathological examination of the tissues revealed no consistent changes associated with any treatment. There was a range of pathological changes in the tissues examined, with the lungs, proventriculus, cecum, and liver being most frequently affected (Table 3). One bird in con-

trol group 1, receiving untreated feed, had mild changes in the cecum in the lower gastrointestinal tract. There were two birds from treatment groups MSP9 and MSP3 that had mild changes in single tissues. Five of the nine birds examined had lesions in at least three and up to seven tissues. The changes, however, were not atypical for normal young pheasants and

TABLE 1B. Comparison of Body Weight Gain (g) and Structural Growth (Tarsal Length, mm) Among Young Pheasants from 9 to 25 d of Age on 10 Different Dietary Treatments Based on Fungal-Infected Grasshopper Food Items, Fungal-Coated (FC) Duckling Starter, Untreated Grasshoppers, or Untreated Duckling Starter (*Continued*)

Dietary treatment	*n*	Sex	Weight gain for 9–17 d of age (0–8 d after treatment)[a] (g)	Weight gain for 17–25 d of age (8–16 d after treatment)[a] (g)	Weight gain for 9–25 d of age (0–16 d after treatment)[a] (g)	Tarsus 25 d of age (16 d after treatment)[a] (mm)
B. Female						
Control-1 Clean grasshoppers	17	F	50.59**ab**	53.64**a**	104.22**a**	47.45**a**
Control-2 FC duckling starter	18	F	48.45**ab**	50.64**a**	99.09**ab**	46.31**ab**
Feed-1 FC duckling starter	18	F	46.73**ab**	46.29**a**	93.01**ab**	45.92**ab**
Feed-2 FC duckling starter	18	F	51.77**a**	51.25**a**	103.02**ab**	48.01**a**
Isolate MSP3-1[b] *M. anisopliae* var. *acridum*	18	F	49.59**ab**	49.63**a**	99.22**ab**	46.94**ab**
Isolate MSP3-2[c] *M. anisopliae* var. *acridum*	18	F	42.06**b**	42.51**a**	84.57**b**	44.20**b**
Isolate MSP9-1[b] *M. anisopliae* var. *acridum*	18	F	47.69**ab**	45.87**a**	93.55**ab**	45.86**ab**
Isolate MSP9-2[c] *M. anisopliae* var. *acridum*	18	F	50.22**ab**	49.81**a**	100.03**ab**	47.04**ab**
Isolate GHA-1[b] *B. bassiana*	18	F	50.91**a**	51.71**a**	102.62**ab**	47.42**a**
Isolate GHA-2[c] *B. bassiana*	18	F	50.82**ab**	54.38**a**	105.20**a**	47.38**a**
Tukey's HSD minimum significant difference (df = 119)			8.85	13.03	18.68	2.89
Scheffé's test minimum significant difference (df = 119)			11.52	16.97	24.33	3.76

Note. Tukey's groupings are used to compare specific treatments. For males, none of the means within columns differed significantly. For females, means with different letter (**a** or **b**) denote groups significantly different from each other at $\alpha = .05$.

[a]Grasshoppers were treated at 7 and 9 d of age.

[b]Grasshoppers dead from entomopathogenic fungal infection.

[c]Grasshoppers dead from entomopathogenic fungal infection and stored 3 d to sporulate at high humidity.

TABLE 2. Summary of Analysis of Variance of Body Weight Gain (g) and Structural Growth (mm) of Pheasant Chicks

Sex	*n*	Variable	$F_{9,45}$	*p* (Greater *F*)
Female	180	Gain to 17 d of age	3.03	.0065
Female	179	Gain from 17 to 25 d of age	1.57	.1520
Female	179	Gain to 25 d of age	2.92	.0082
Female	179	Tarsus, mm	4.76	.0002
Male	180	Gain to 17 d of age	1.02	.4400
Male	179	Gain from 17 to 25 d of age	1.37	.2294
Male	179	Gain to 25 d of age	1.52	.1685
Male	179	Tarsus, mm	0.73	.6800

could not consistently be ascribed to either the challenged or the control groups.

No fungal colonies other than *Mucor* and *Penicillium* were obtained from plating the selective media with feces and blood from treated birds. However, plating samples of the diet yielded lower numbers of colony forming units (CFUs) than expected based on measured germination rates, so absence of viable fungus in the birds' body fluids is not conclusive.

Effects of Consumption of Spores

Pheasants in the treatments provided with spores of feed did not have higher mortality or lower weight gain than control birds, nor did they exhibit symptoms of illness.

DISCUSSION

Little evidence for lethal impacts of entomopathogenic agents on homeothermic vertebrates has been reported in previous investigations, although activity of the mycotoxin beauvericin on mammalian tissue has been described (Nakajyo et al., 1987). Respiratory distress was reported in test mammals inhaling *B. bassiana* conidia (Müller-Kögler, 1967), while weight loss and some mortality was reported in rats after oral exposure to conidia and mycelium, but details of exposure were not given (Schaerffenberg, 1968). Young kestrels fed *B. bassiana* GHA conidia suffered no demonstrable adverse effects (Althouse et al., 1997). Disease has been described in rare cases in exposed ectotherms, mainly reptiles and amphibians. *Beauveria bassiana* has caused fatal lung infections in three captive giant tortoises (*Testudo nigra,* Bour, 1984; *T. gigantea elephantina*) at the Chicago Zoological Park (Georg et al., 1962), and in two captive American alligators (*Alligator mississipiensis*) (Fromtling et al., 1979a, 1979b). In general, mycotic infections are likely to occur in captive reptiles only when subjected to inadequate management and undesirable environmental conditions (Zwart, 1986).

Unlike the generally more temperature-sensitive *B. bassiana, M. anisopliae* var. *acridum* is capable of growth and survival under conditions near

mammalian and avian body temperatures. In safety tests of the impact of pure conidia of *M. anisopliae* var. *acridum,* no serious clinical signs were observed in mice, rats, and rabbits (Sherwood et al., 1994), except in inhalation tests with extremely high doses of spores. Experiments in Niger indicated no significant impact on survival of 2-mo-old Rhode Island Red chickens, consuming pure spores on feed provided ad libitum (Kooyman, 1995 unpublished report, DFPV, Niamey, 23/01/95; cited in Lomer, 1996; pp. 25–26). As was the case in our study, no gross pathology or change in organ weight was seen in the alimentary tract, liver, heart, or lungs during the pathologist's examination of 10 of the treated birds. Most other tests of avian toxicity (e.g., quails and *M. anisopliae* var. *acridum* conidia; Hartmann & Wasti, 1980; Wasti et al., 1980) have been conducted using spores, but not infected insect food items, as has been done in this study.

As noted by Prior (1990), there is generally little evidence of toxicity to vertebrates from most of the compounds produced by entomopathogenic fungi. It is likely that conditions within the tissues of homeothermic animals do not allow fungal development that would be required for the elaboration of mycotoxins, or successful invasion by the fungal hyphae.

Screening for pathological impact on tissues of these experimental birds was necessary for two reasons. Direct tissue invasion or pathology due to exposure to fungal-infected food items could occur. In addition, mycotoxins,

TABLE 3. Histopathology Scores of the Sample of Birds Examined

	Bird ID number, treatment							
Tissue	4–51, Control-1	4–59, Control-2	3–34, GHA-1	4–37, GHA-2	4–32, MSP9-1	4–31, MSP9-2	4–43, MSP3-1	4–36, MSP3-2
Spleen	2	1	1	1	1	1	1	3
Bursa	1	1	2	1	1	1	1	1
Thymus	1	1	1	1	3	1	1	A
Lung	2	1	2	1	1	2	1	1
Crop	1	1	1	1	1	1	1	A
Proventriculus	2	1	2	2	2	1	1	1
Gizzard	1	1	1	1	1	1	1	1
Duodenum	1	1	2	A	2	1	1	1
Pancreas	1	1	1	1	2	1	1	1
Jejunum	2	1	A	A	1	A	1	A
Cecum	1	2	1	2	2	1	1	2
Colon	1	1	2	1	A	1	1	A
Kidneys	1	1	1	2	2	1	1	2
Myocardium	1	1	1	1	1	1	1	1
Liver	1	1	2	2	2	1	3	1
Brain	1	1	1	1	1	1	1	1

Note. 1, normal or with mild artifactual tissue changes; 2, one or two small lesions, mild pathological changes; 3, numerous mild or at least one large lesion; 4, indications of extensive pathological change; A, sample absent, or unable to obtain adequate tissue.

the intermediate metabolites produced by many fungi, could cause histologically detectable tissue damage (Scott et al., 1985). For example, T-2 toxin, a trichothecene metabolite of *Fusarium*, produces immunotoxicity and cytotoxicity (Bondy & Pestka, 2000), as well as neurotoxicity, cardiotoxicity, and teratogenesis (Reddy & Hayes, 1989). Destruxins, cyclodepsipeptide toxins elaborated by the entomopathogenic fungus *Metarhizium anisopliae* var. *acridum* (Gupta et al., 1989; James et al., 1993), cause cytotoxic effects to cytoplasmic organelles in insect hosts (Vey & Quiot, 1989). Oosporein is a mycotoxin that can be produced by *B. bassiana*. Pathology such as gizzard mucosal necrosis, proventricular inflammation, articular and muscular urate deposits, and hepatic and renal inflammation has been described in young chickens orally exposed to oosporein (Pegram & Wyatt, 1981; Manning & Wyatt, 1984). In the present study with young pheasants, there is no evidence of similar tissue damage, suggesting that oosporein production was not a concern in birds eating either infected insects or the conidial stages of the isolates of entomopathogenic fungi *B. bassiana* and *M. anisopliae* var. *acridum* under study here.

In avian species, negative environmental factors such as infectious, nutritional, or physical stress result in glucocorticoid release and subsequent B- and T-lymphocyte death or apoptosis. This may result in bursal or thymic atrophy, with increased evidence of apoptosis throughout the tissue (Pope, 1996). If the entomopathogenic fungi behaved as infectious agents, they would be expected to provoke an immune response in the birds, which would be reflected in changes in the primary (thymus and bursa) and secondary (spleen) organs of immunity. These were not present.

Food avoidance and emesis that have been described in animals exposed to fungal metabolites (Mirocha & Christensen, 1974) were not seen in this study. Except in a few cases in which insects were advanced in fungal sporulation, the birds fed readily on infected insects and showed no detectable signs of subsequent avoidance or ill health, implying that neither the mycotic agents nor potential mycotoxins were problematic. The mild pathological changes that were seen in the birds in this study likely represented a normal range of incidental pathological changes in a group of young birds being raised in a captive, indoor environment.

The data presented here comprised part of the body of research that resulted in *M. anisopliae* var. *acridum* obtaining approval for production for locust control by the Malagasy Government (decree 99-798, 6 October 1999).

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