BALANCING SELECTION FOR AFLATOXIN PRODUCTION IN *ASPERGILLUS* FLAVUS MAY BE MAINTAINED BY COMPETITION WITH INSECTS AND MICROBES

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Milton Thury Drott

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BALANCING SELECTION FOR AFLATOXIN PRODUCTION IN *ASPERGILLUS* FLAVUS MAY BE MAINTAINED BY COMPETITION WITH INSECTS AND MICROBES

Milton Thury Drott, Ph.D.

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The role of microbial secondary metabolites in the ecology of the organisms that produce them remains poorly understood. Variation in aflatoxin production by *Aspergillus flavus* is maintained by balancing selection, but the ecological function and impact on fungal fitness of this compound are unknown. I hypothesize that balancing selection for aflatoxin production in *A. flavus* is driven by interaction with insects and microbes. To test this, I competed naturally occurring aflatoxigenic and non-aflatoxigenic fungal isolates against drosophila larvae and soil microbes using microcosm experiments. In all microcosms I used quantitative PCR to quantify DNA as a proxy for fitness. I demonstrate that, in the presence of insects, aflatoxigenic isolates have a fitness advantage relative to non-aflatoxigenic isolates. I speculate that this advantage is conferred through interference competition and protection from fungivory. Conversely, I demonstrate that aflatoxigenic isolates have a fitness cost relative to non-aflatoxigenic isolates when competing with soil microbes. I speculate that this fitness cost is the result of energetic costs associated with aflatoxin production. Using field isolates from two north-south transects spanning the United States, I demonstrate that patterns in the frequency of aflatoxigenic isolates are consistent with selection at a local level. To the best of my knowledge, this work represents the first evidence for possible selective pressures driving balancing selection and the most comprehensive assessment of *A. flavus* population structure in the United States to date.

BIOGRAPHICAL SKETCH

Milton T. Drott graduated from Franklin and Marshall college with a BA in biology in 2011. During his tenure at Franklin and Marshall he attended an REU at the Harvard Forest where he was introduced to the impact of plant pathogens on natural landscapes. After spending a year working on bacterial endosymbionts of aphids at Drexel University, Milton joined Michael Milgroom's lab in the department of Plant Pathology and Plant-Microbe Biology at Cornell University in 2013.

To my mother, Eva Thury, father, Carl Drott, and cat, Penny, in gratitude for their unwavering support.

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CHAPTER 1

Balancing selection for aflatoxin in *Aspergillus flavus* is maintained through interference competition with, and fungivory by insects.

Milton T. Drott[1], Brian P. Lazzaro[2], Dan L. Brown[3], Ignazio Carbone[4], Michael G. Milgroom[1]

1. School of Integrative Plant Science, Plant Pathology and Plant-Microbe Biology Section, Cornell University, Ithaca, NY 14853

2. Department of Entomology, Cornell University, Ithaca, NY 14853

3. Department of Animal Science, Cornell University, Ithaca, NY 14853

4. Center for Integrated Fungal Research, Department of Entomology and Plant Pathology, North Carolina State University, Raleigh, NC 27695

Key Words:
Balancing selection, aflatoxin, fungivory, interference competition, secondary metabolism, mycotoxins

Abstract:

The role of microbial secondary metabolites in the ecology of the organisms that produce them remains poorly understood. Variation in aflatoxin production by *Aspergillus flavus* is maintained by balancing selection, but the ecological function and impact on fungal fitness of this compound is unknown. We hypothesize that balancing selection for aflatoxin production in *A. flavus* is driven by interaction with insects. To test this, we competed naturally occurring aflatoxigenic and non-aflatoxigenic fungal isolates against *Drosophila* larvae on medium containing 0-2000 ppb aflatoxin, using qPCR to quantify *A. flavus* DNA as a proxy for fungal fitness. The addition of aflatoxin across this range resulted in a 26-fold increase in fungal fitness. With no added toxin, aflatoxigenic isolates caused higher mortality of

Drosophila larvae, and had slightly higher fitness than non-aflatoxigenic isolates. Additionally, aflatoxin production increased an average of 1.5-fold in the presence of a single larva and nearly 3-fold when the fungus was mechanically damaged. We argue that the role of aflatoxin in protection from fungivory is inextricably linked to its role in interference competition. Our results provide the first clear evidence of a fitness advantage conferred to *A. flavus* by aflatoxin when interacting with insects.

Introduction:

Despite recognition in the literature of the vast diversity and prevalence of microbial secondary metabolites (Davies and Davies 2010), the role of these compounds in the ecology of the organisms that produce them remains poorly understood. In the case of antibiotics, an anthropomorphic perspective has led to the dogma that because a molecule may have clinical or laboratory efficacy against bacteria, it is in fact produced for the purpose of mediating antagonistic interactions in natural habitats (Yim, Huimi Wang et al. 2006). Gould and Lewontin (1979) warned that such misappropriations of current utility for inferring evolutionary origin create an unproductive conceptual architecture in the literature. While there is some evidence of antibiotic production increasing microbial fitness through inhibition of other bacteria (Chao and Levin 1981), it is now commonly thought that subinhibitory concentrations are the norm (Allen, Donato et al. 2010). At these concentrations, many 'antibiotics' have exhibited hormesis. Antibiotic targets of inhibition are now seen as signaling receptors (Davies, Spiegelman et al. 2006, Ryan and Dow 2008, Allen, Donato et al. 2010). Although work on antibiotics as signaling molecules has been extensively

reviewed (Davies, Spiegelman et al. 2006, Yim, Huimi Wang et al. 2006, Ryan and Dow 2008, Davies and Davies 2010), new hypotheses proposing the evolutionary origin of antibiotics continue to emerge.

Even with such limited understanding of microbial secondary metabolism in general, there have been significant developments in our knowledge about fungi in this respect. Genomic analysis of a wide range of compounds, including mycotoxins, has shown how the regulation of secondary metabolism functions (reviewed by Keller, Turner et al. 2005, Yu and Keller 2005, Fox and Howlett 2008, Macheleidt, Mattern et al. 2016). A compelling example of an adaptive function of a single fungal secondary metabolite is found in the study of deoxynivalenol (DON) produced by *Fusarium graminearum*. Although DON-non-producing mutants can initiate infection, their virulence on a wheat host is dramatically reduced (Bai, Desjardins et al. 2002). Nevertheless, as with antibiotics, connecting functionality in the lab to adaptive hypotheses about secondary metabolism has proven difficult. Part of this difficulty is in separating the potential benefit of a small molecule from other pleiotropically linked fitness traits (Calvo, Wilson et al. 2002). Such linkages are especially problematic when studies use mutants of secondary metabolism regulators that may impact the production of many compounds. In *Aspergillus spp.*, for example, *laeA* is a gene described as a global regulator of secondary metabolism. *Aspergillus flavus* mutants with this gene deleted (Δ*laeA* mutants) exhibit significantly decreased aflatoxin, spore and sclerotial production (Kale, Milde et al. 2008). In addition, the expression of other secondary metabolites is affected by this regulatory gene (reviewed by, Bok and Keller 2016). Despite these advances in understanding the

regulation of fungal secondary metabolites, there remains a need for further study of their impacts in general on the ecology of organisms that produce them (Rohlfs and Churchill 2011).

Recently, several studies have attempted to show the ecological role or selective forces driving the evolution of fungal secondary metabolism, many focusing on their potential interaction with insects. Many of these studies have used mutants like Δ*laeA* that affect the production of multiple secondary metabolites. For example, Trienens et al. (2010) showed that Δ*laeA* mutants of some *Aspergillus* spp. had slower growth than wild type in culture when confronted with *Drosophila* larvae. Other studies have also provided evidence for a role of fungal secondary metabolism in competition with insects (Staaden, Milcu et al. 2010, Trienens and Rohlfs 2011, Trienens and Rohlfs 2012). In addition, some specific fungal compounds may have a direct role in inhibiting fungivory (Döll, Chatterjee et al. 2013, Ortiz, Trienens et al. 2013). However, the use of mutants like Δ*laeA* to address this type of question may conflate the effects of multiple secondary metabolites, making it impossible to understand the ecological role of a specific compound.

Few secondary metabolites have received as much attention as the mycotoxin aflatoxin, produced by *A. flavus, A. parasiticus* and a few other *Aspergillus* species in section *Flavi*. Aflatoxin is an extremely potent hepatotoxin that causes acute toxicosis, cancer, immune suppression, and stunted growth in children (Williams, Phillips et al. 2004, Wild 2007, Liu and Wu 2010, Wild and Gong 2010). However, not all strains of *A. flavus* produce aflatoxin. Extensive field sampling of *A. flavus* in the US found that 29% of all isolates were non-aflatoxigenic (Horn and Dorner 1999). Worldwide, both

chemotypes (aflatoxigenic and non-aflatoxigenic) are often found in the same field (Horn 2003). Moreover, nucleotide sequence analysis of 21 regions in the aflatoxin gene cluster in *A. flavus* and *A. parasiticus* indicated that polymorphism for aflatoxin production is maintained by balancing selection (Carbone, Jakobek et al. 2007). However, the selective forces that drive the balancing selection for aflatoxin production have remained a mystery.

Janzen (1977) elaborated the hypothesis that aflatoxin production is favored in the presence of soil microbes, birds, mammals or insects with which the fungus engages in interference competition. Under this hypothesis, the toxic effects of aflatoxin produced in nutrient-rich substrates, such as seeds, increases fungal fitness by deterring competitors. Implicitly, when these competitors are absent, the cost of toxin production favors non-producers, thereby driving balancing selection. In the decades since, the amount of research on insect-aflatoxin interactions has eclipsed research on effects to the other potential competitors. Many studies have demonstrated that pure aflatoxins added to food sources are toxic to a wide range of insects (Racovitza 1969, Niu, Wen et al. 2008, Rohlfs and Obmann 2009, Staaden, Milcu et al. 2010), although the degree of toxicity varies greatly even within a genus (Rohlfs and Obmann 2009). However, evidence of toxicity of aflatoxin to insects without evidence of increased fungal fitness does not serve to explain balancing selection acting on the fungus for aflatoxin production. Wicklow et al. (1994) speculated that the increasing toxicity to the European corn borer of compounds along the aflatoxin biosynthetic pathway (Jarvis, Guthrie et al. 1984) is consistent with an evolutionary arms race against an insect immune system. Works like these provide a conceptual framework for the

hypothesis that aflatoxigenic individuals may have greater fitness in the presence of insects whereas non-aflatoxigenic individuals may be favored in their absence, thus maintaining the polymorphism for aflatoxin production. Despite ample evidence that aflatoxins are toxic to insects, direct demonstration that aflatoxin production affects fungal fitness is lacking.

In the present study, we determined the effect of aflatoxin on fungal fitness using a modified version of the *Aspergillus/Drosophila* model system used by Trienens et al. (2010). We compared naturally occurring strains of *A. flavus* that produce aflatoxin with those that do not to avoid complications associated with using laboratory mutants when studying fitness. Our study thus aims to determine whether interaction with insects, regardless of mechanism, can account for balancing selection acting on aflatoxin production in *A. flavus*. Specifically we addressed the following questions: 1) Does aflatoxin decrease the fitness of *Drosophila* when it is added directly to food? 2) Does aflatoxin increase the fitness of *A. flavus* in the presence of *Drosophila* larvae when it is added to a nutrient source? 3) Do aflatoxigenic isolates of *A. flavus* have higher fitness compared to naturally occurring non-aflatoxigenic isolates when interacting with *Drosophila*? 4) Does physical damage by *Drosophila* larvae to *A. flavus* result in an increase in aflatoxin production?

Methods:

Cultures of *Aspergillus flavus* and *Drosophila*:

Field isolates of *A. flavus* used in experiments were obtained previously (table S1). Cultures were revived from lyophilized mycelium stored at -80 °C and grown on

Czapek-Dox agar at 30 °C in the dark for 5 d. Spores were harvested in sterile deionized H_2O (diH_2O) with 0.05% tween 20 and counted on a hemocytometer. Although *A. flavus* has the potential to produce a variety of mycotoxins, for example, cyclopiazonic acid (CPA), whose biosynthetic cluster is adjacent to the aflatoxin cluster (Moore et al. 2009), in this paper we refer to 'toxigenic' and 'non-toxigenic' isolates based solely on their ability to produce aflatoxin.

A *Drosophila melanogaster* population of the strain Canton-S was used for all experiments. Flies were maintained at room temperature on medium containing 50 g yeast, 70 g yellow cornmeal, 40 g glucose, 7 g agar, per L diH_2O, plus 1 ml of a solution containing 4.2% phosphoric acid and 42% propionic acid to control microbial growth. To harvest *Drosophila* larvae for the experiments below, adult flies were transferred to fresh medium and allowed to lay eggs for 16 h. Resulting eggs were removed using an artist's paintbrush and sterilized in 0.25% sodium hypochlorite for 10 min, rinsed with sterile diH_2O, and transferred to 3% water-agar plates. Hatched larvae were transferred to experimental tubes 16 h later.

Experimental Microcosms:

All experiments were conducted in 2-mL microcentrifuge tubes containing 200 µl of a modified *Drosophila* culture medium (DCM) (Trienens, Keller et al. 2010). The medium contained 57.2 g sucrose, 57.2 g sieved cornmeal (particles <0.25 µm in size), and 57.2 g brewer's yeast per L DiH_2O. After the medium had solidified in the bottom of the tube, a sterile tooth pick was used to macerate it, increasing surface area for greater colonization by *A. flavus*. Without maceration, we found that the burrowing of larvae greatly increased the surface area of the medium resulting in greater fungal

fitness relative to tubes without larvae (no-larvae controls). Tubes containing macerated medium were randomly assigned to 'larvae' or 'no-larvae' treatments. Nine first-instar *Drosophila* larvae were transferred with a paintbrush to each tube assigned to the larvae treatment (in Experiment 4 tubes received only a single larva). Fungal isolates were randomized into resulting larvae and no-larvae tubes and 5 µl of 70 spores/ µl solution was placed on the surface of the medium; the same volume of diH_2O was added to no-fungus controls. Tubes were plugged with sterile cotton and randomized to positions on a rack. Racks were kept in a loosely sealed clear-plastic bin with water in the bottom to maintain high humidity and avoid drying of the medium. Bins were maintained on a 12-hour dark/light cycle under a fluorescent lamp on a lab shelf at room temperature. After incubation, three replicate tubes of the same fungal isolate/larval treatment were randomly assigned to assays of fungal fitness, aflatoxin content, or fly fitness. In preparation for DNA and aflatoxin extractions, the contents of experimental tubes were frozen and lyophilized. In Experiment 4, fungal fitness and aflatoxin content were assayed from the same tube, as described below.

HPLC for quantifying aflatoxin

For a given treatment, the lyophilized contents of three microcosm tubes were combined in a 2-ml microcentrifuge tube containing zirconia-silica beads of 2.5 mm and 1 mm diameters, and 1 mL of 80% MeOH was added. Tissue was homogenized on the Thermo Savant Bio101 Fast Prep 120 (Qbiogene, Carlsbad, CA, USA) set to 6.5 for 45 seconds. Resulting suspensions were centrifuged at 13,800 g for 8 min and 450 µl of aflatoxin extract was combined with 1440 µl of DiH2O to achieve 25%

MeOH. This suspension was filtered through 0.4 μm syringe filters into silanized autosampler vials.

We determined later that 750 μl of DNA extraction buffer (see below) in addition to 750 μl of chloroform would allow for extraction of aflatoxin B1 and DNA from the same tube. This method was used for Experiment 4. After the removal of aqueous phase, 500 μl of chloroform was transferred to a silanized tube and dried. Aflatoxin B1 was resuspended in 1 ml of 25% MeOH and filtered as described above. All aflatoxin measurements were corrected for dilution.

Aflatoxin B1 was quantified on an Agilent 1100 HPLC (Santa Clara, CA, USA) in comparison with a standard curves constructed from analytical standards (Sigma Aldrich CRM44647, St. Louis, MO, USA) diluted to 25% MeOH in the range of 8 ng/μL-0.0008 ng/μl (8000 ppb-0.8 ppb). All standard curves were linear across this range. HPLC runs were performed using 45% methanol as the mobile phase across a Zorbax Eclipse XDB-C18 Analytical 4.6x250 mm column (Agilent Technologies, Santa Clara, CA, USA). Injection of 50 μl was run at 0.6 ml/min at 45 °C for 15 min. Resulting peaks were integrated and converted to ng/ml reported as parts per billion (ppb) by comparison to standard curve constructed using analytical grade aflatoxin B1. As we only quantified aflatoxin B1, all subsequent references to 'aflatoxin' refer this form of the toxin.

Quantitative PCR (qPCR) for estimating fungal fitness

Lyophilized contents of microcosm tubes were transferred to corresponding bead-beating tubes as described above for aflatoxin extraction. DNA was extracted as described previously (Kepler, Humber et al. 2014). After adding 1 ml of extraction

buffer, samples were homogenized in a Fast Prep 120 set to 6.5 for 45 seconds. Tubes were then centrifuged at 13,800 g for 8 min and 600 µl of supernatant was removed to a clean tube. An equal volume of phenol:chloroform:isoamyl alcohol (24:8:1) was added and samples were vortexed for 10 seconds before they were centrifuged again at 18,000 g for 5 min. Supernatant (100 µl less than the previous step) was again mixed and centrifuged with an equal volume of phenol:chloroform:isoamyl alcohol (24:8:1). This step was repeated four times and once more using an equal volume of chloroform:isoamyl alcohol (24:1). The supernatant from this last wash, a final volume of 100 µl, was moved to a clean 2-ml tube where 10 µl of 3M NaOAc (pH 5.5) was added and mixed by vortexing for 5 seconds. DNA was precipitated in two volumes of -20 °C ethanol by vortexing for 5 seconds and storing the resulting suspension at -20 °C for 16 h. DNA was pelleted by centrifugation at 10,000 g for 10 min and ethanol was decanted. Pellets were washed with 400 µl of 70% ethanol (4 °C) for 10 seconds and centrifuged again at 18,000 g for 5 min. After decanting the ethanol, pellets were air dried upside down on a paper towel for 5 min. DNA was suspended in 200 µl of TE.

We developed qPCR primers by aligning sequences of the O-methyltransferase gene (*omtA-1*) of the aflatoxin biosynthesis cluster sequences from GenBank (Accession numbers, table S2) using ViiA7 (Thermo Fisher) to generate potential primers. The resulting primer pair, AflO8-F 5’ AGTGACAGAGCGTCCGAATC and AflO8-R 5’ GGCGGTGACGATGTTAGAGA, produces an amplicon of 73 bp. Melt-curve and gel-electrophoresis analyses were conducted using DNA extracts from microcosms with and without *A. flavus* or *Drosophila* larvae. Template controls lacking *A. flavus*

did not ever show amplification. This genetic marker was further validated by comparing a random sample of fitness estimates based on results from the Aflo8 primers to those from primers we developed for the *A. flavus* actin gene (*Act1*) (see Supplemental Methods). Results from the two primer sets were highly correlated (r=0.966).

qPCR reactions were run in triplicate on a CFX-Connect Real-Time Detection system (Bio-Rad, Hercules, CA, USA) using default settings. Each 25-µl reaction contained 12.5 µl of SsoAdvanced SYBR green supermix, forward and reverse primers at 0.1 µM, 2 µl DNA template, and 8 µl ultrapure water. PCR cycling conditions were: 95 °C for 5 min, 40 repeats of 95 °C for 20 sec, 64.2 °C for 30 sec. Melt-curve analysis was done in 0.5 °C increments between 60 and 95 °C after 10 sec at 95 °C.

A. flavus DNA was quantified against a standard curve constructed for each experiment. DNA used for constructing the standard curve was extracted from no-larvae microcosms and was diluted in a 10-fold dilution series (2×10^{0} to 2×10^{-4}). Dilutions were aliquoted into tubes stored at -20 °C to allow for comparison of subsequent experimental DNAs without additional freeze-thaw steps. All standard curves indicated 93-99% efficiency with r^2 values >0.99. Experimental DNA was diluted by 1×10^{-1} before analysis; qPCR results were corrected for dilution. DNA was used as a proxy for fungal fitness and thus each experiment resulted in fungal fitness relative to a single standard curve. Standard curves were identical for all plates within an experiment, but were not comparable between experiments.

Experiment 1: Effect of aflatoxin on *Drosophila* fitness:

To test the hypothesis that aflatoxin reduced the fitness of *Drosophila*, we added

aflatoxin ranging from 0-4000 ppb in increments of 500 ppb to the food in experimental tubes. When applicable, aflatoxin B1 (Sigma Aldrich A6636) dissolved in methanol was added to molten DCM after autoclaving. Aflatoxin was diluted further in methanol to standardize the amount of methanol added, regardless of aflatoxin treatment. At each concentration, nine microcosms (N=81) were observed microscopically every 24 h for 15 d. The number of pupated or emerged flies was recorded.

The number of pupae and the logarithm of the number of emerged flies observed in a given tube after 15 d were analyzed using a simple linear regression against the aflatoxin content of food. Emergence data were $\log_{10}$ transformed because of unequal variance in the arithmetic scale.

Experiment 2: Effect of aflatoxin on fitness of *Aspergillus flavus* in the presence of *Drosophila* larvae:

To look at the role of aflatoxin on fitness of *A. flavus,* we used microcosms containing 0, 525, 1050, 1750 ppb aflatoxin. Six field isolates of *A. flavus* (three aflatoxigenic and three non-aflatoxigenic) were grown on medium at all four concentrations. The design was fully factorial, with larvae and no-larvae treatments. Resulting microcosms were incubated for 72 h. We processed three tubes for each isolate for aflatoxin (see above) and three for DNA for qPCR (see above), with and without flies at each concentration of aflatoxin (N=288).

Additionally, three tubes per fungal isolate and three no-fungus control tubes were used at every aflatoxin concentration to assess *Drosophila* survival in the presence of both the fungus and the added toxin (N=84). In order to allow for some larvae to begin

pupation, facilitating the differentiation of *Drosophila* life stage, these tubes were incubated for a total of 96 h. *Drosophila* fitness was determined by examining tubes under a dissecting microscope (3-30×). To dislodge the food and the insects from the bottom of the tube, 500 µl of DiH2O was added and vortexed for 5 s. The tube contents were deposited onto a petri dish. The resulting slurry was diluted further with water and dissected using forceps. Given the difficulty of finding dead *Drosophila* larvae in medium colonized by the fungus, only living *Drosophila* were counted; the rest were presumed dead.

A mixed linear model was constructed to explain differences in fungal fitness as a function of main and interaction effects of: the quantitative variable of toxin added to the food, the qualitative variable of larvae/no-larvae, and the qualitative variable of fungal aflatoxigenicity nested in the random effect of fungal isolate. Pairwise differences were also explored at specific food-aflatoxin levels using a Tukey post hoc test.

Experiment 3: Effect of aflatoxin on fitness of *Aspergillus flavus* in the presence of *Drosophila* larvae:

We used nine toxigenic and nine non-toxigenic field isolates of *A. flavus* to determine whether production of aflatoxin conferred a fitness benefit in the absence of adding exogenous aflatoxin as done in previous experiments. The design was fully factorial, with larvae and no-larvae treatments. DNA from three tubes was pooled, whereas aflatoxin extracts were sampled from three separate tubes as described above.

A mixed linear model was constructed to explain differences in square-root fungal fitness as a function of the qualitative variable of aflatoxigenicity nested in the random

effect of fungal isolate. The square-root of fungal fitness was used to equalize variances and linearize the relationship between response and predictor variables. Toxigenic and nontoxigenic isolates were compared within larvae and within no-larvae treatments.

Experiment 4: Effect of physical damage and feeding by *Drosophila* on aflatoxin production:

We used 12 toxigenic field isolates of *A. flavus* to assess whether aflatoxin production was increased because of feeding by *Drosophila* larvae and whether this effect was different from that of physical damage. Each isolate was replicated three times in three treatments: larva, no-larva, or physical damage. Larva tubes received a single larva in the same manner as described above. At 24, 36, 48 and 60 h cultures of *A. flavus* in the physical damage treatment were stabbed 30 times each with the tip of a round toothpick. This method is similar to that used by Ortiz et al. (2013). Resulting damage to colonies resembled damage observed when larvae were present. The other two treatments also had their cotton plugs removed and replaced at the same times as the physical damage treatment but were otherwise left undisturbed. At 72 h DNA and aflatoxin were extracted from the same microcosms as described above.

A mixed linear model was constructed to explain differences in $\log_{10}$(aflatoxin) as a function of the main and interaction effects of fungal fitness and treatment. Pairwise differences were also explored using a Tukey post hoc test. Aflatoxin concentrations were $\log_{10}$ transformed to normalize residuals and linearize relationship between response and predictor variables.

General Statistical Methods:

Results were analyzed using R statistics 3.4.0 (R Core Team 2017) Packages 'lme4' (Bates, Mächler et al. 2015) , 'car' (Fox and Weisberg 2011), 'lmerTest' (Kuznetsova, Brockhoff et al. 2016), and 'lsmeans' (Lenth 2016), 'tidyverse' (Wickham 2017), and 'Rmisc' (Hope 2013) installed on 21 April 2017.

Results:

Experiment 1: Effect of aflatoxin on *Drosophila* fitness:

Aflatoxin significantly ($P<0.0001$) decreased the number of *Drosophila* pupae that formed (figure 1A) as well as the number of adult flies that emerged (figure 1B). Although a small number of pupae formed at concentrations of 2500 and 3000 ppb, none of these resulted in emerged adult flies. At higher aflatoxin concentrations, larvae were much less likely to burrow through food as was evident from the undisturbed surface-relief of the medium without maceration. This suggests that the insects did not consume the food at higher aflatoxin concentrations. Melanization (figure S1), indicating a response to tissue damage, was more commonly observed at intermediate aflatoxin concentrations where the larvae were still feeding on the medium than at high concentrations where feeding was minimal (results not shown).

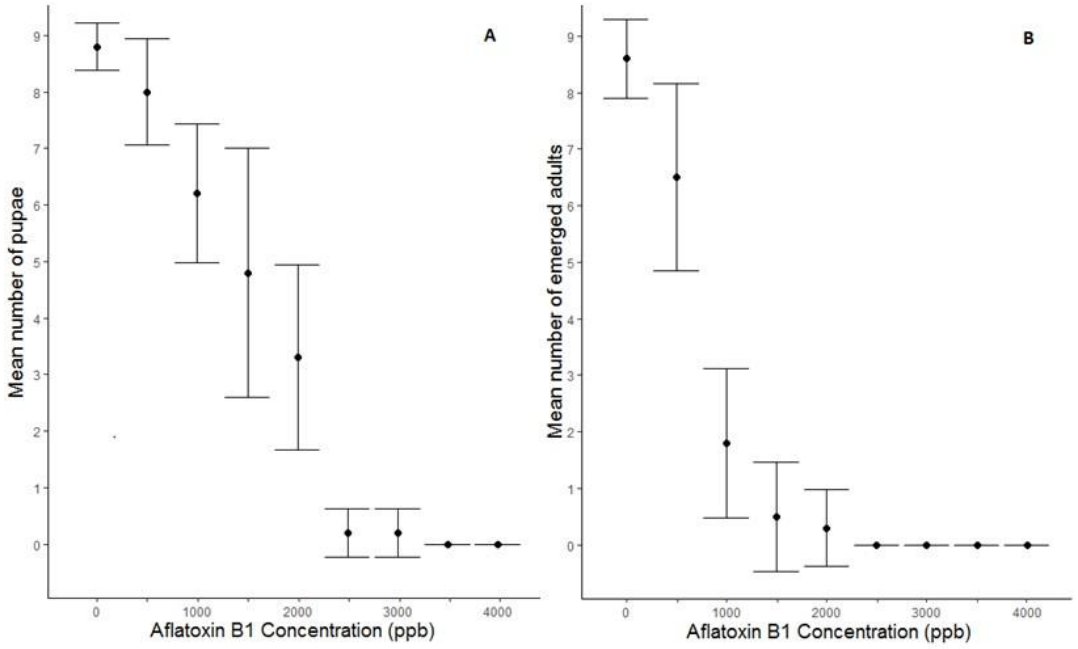

Figure 1. Dose-response curves of *Drosophila* fitness as a function of aflatoxin B1 added to the medium. The number of pupated larvae (A) or subsequently emerging adult flies (B) was tallied over 15 d. All points are the average of 10 replicated tubes, each containing nine larvae at a given aflatoxin concentration (N=90). Error bars represent ± one standard deviation. Aflatoxin significantly decreased both pupation and emergence of larvae (P<0.0001).

Experiment 2: Effect of aflatoxin on fitness of *Aspergillus flavus* in the presence of *Drosophila* larvae:

Increasing concentrations of aflatoxin in the food had a highly significant impact on fitness of *A. flavus* as a function of the presence or absence of *Drosophila* larvae (P<0.0001). In the presence of larvae, average fitness increased linearly almost 26-fold between 0 ppb aflatoxin and 1750 ppb (figure 2). When flies were not present, however, there was no change in fitness of *A. flavus* as aflatoxin concentration of the medium increased. This difference between fitness with and without larvae present was highly significant (P<0.0001) except at 1750 ppb (P=0.2066). There was,

however, no difference in fitness between toxigenic and non-toxigenic isolates in the same larvae/no-larvae treatments (P=0.2664).

In this same experiment, larval survival in the presence of the fungus varied as a function of the interaction between aflatoxin concentration in food and the aflatoxin-producing ability of fungal isolates (P<0.0001) (figure 3). With no aflatoxin added, toxigenic isolates caused significantly lower survivorship in *Drosophila* than non-toxigenic isolates (P=0.0186) and no-fungus controls (P=0.0051). However, there was no difference in larval survivorship between non-toxigenic and no-fungus controls (P=0.1473) when no aflatoxin was added. As aflatoxin concentrations increased, larval survival decreased linearly relative to controls in toxigenic and non-toxigenic fungal treatments, until few or no flies survived in either fungal treatment at the highest aflatoxin concentrations (figure 3).

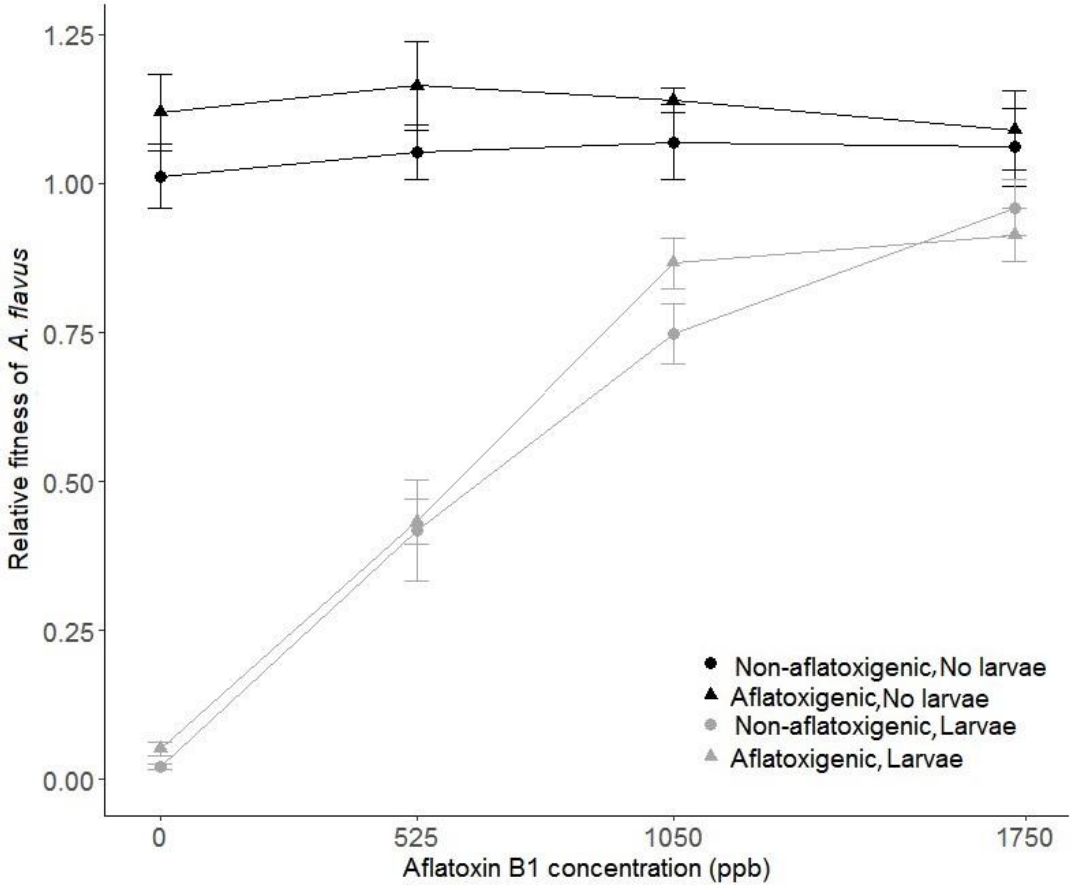

Figure 2. Average relative fitness of three aflatoxigenic and three non-aflatoxigenic isolates of *Aspergillus flavus* with and without *Drosophila* larvae incubated for 72 h. Fitness of *A. flavus* was estimated by qPCR. Each isolate was replicated three times with and without *Drosophila* larvae at each aflatoxin concentration (N=144). Error bars represent ±SE. Fitness did not differ between aflatoxigenic and non-aflatoxigenic isolates (P=0.2664). Fitness was, however, greater in no-larvae treatments than in larvae treatments (P<0.0001) except at 1750 ppb aflatoxin concentration (P=0.2066).

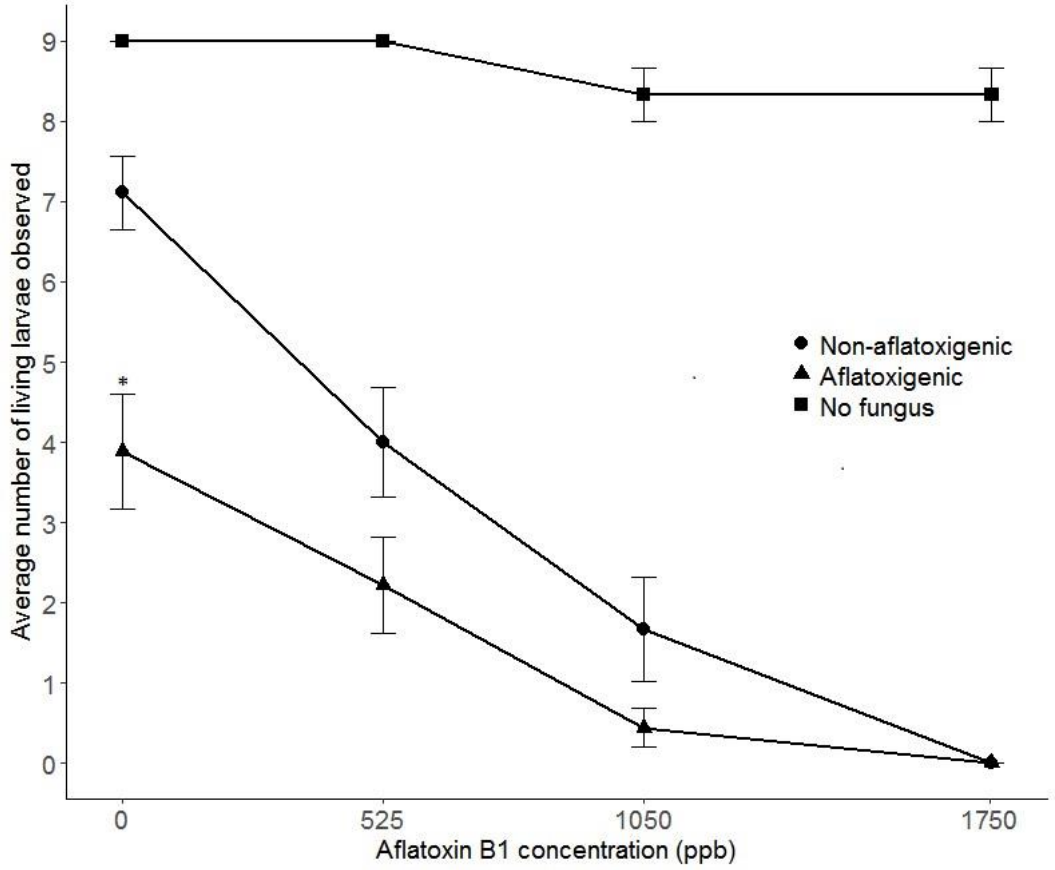

Figure 3. Average number of living larvae observed in microcosms after 96 h with either aflatoxigenic, non-aflatoxigenic fungus or no *Aspergillus flavus*. Means are based on three independent replicates of each of three isolates in each fungal treatment or three replicates of no-fungus treatment for each aflatoxin concentration (N=84). Error bars represent ±SE. With no aflatoxin added, significantly fewer living larvae were observed in aflatoxigenic isolate treatments (indicated by '*') than non-toxigenic isolates (P=0.0186) and no-fungus controls (P=0.0051).

Experiment 3: Effect of aflatoxigenicity in *A. flavus* in the presence of *Drosophila* larvae:

We found no significant interaction determining fitness of *A. flavus* between fungal aflatoxin-producing ability and the presence of flies (P=0.849). There was no

significant difference in fitness between toxigenic isolates and non-toxigenic isolates in the absence of flies (*P*=0.17). When flies were added, fitness was slightly higher for toxigenic isolates than for non-toxigenic isolates (*P*=0.033 for one-sided hypothesis test that toxigenic isolates have greater fitness than nontoxigenic) (figure 4).

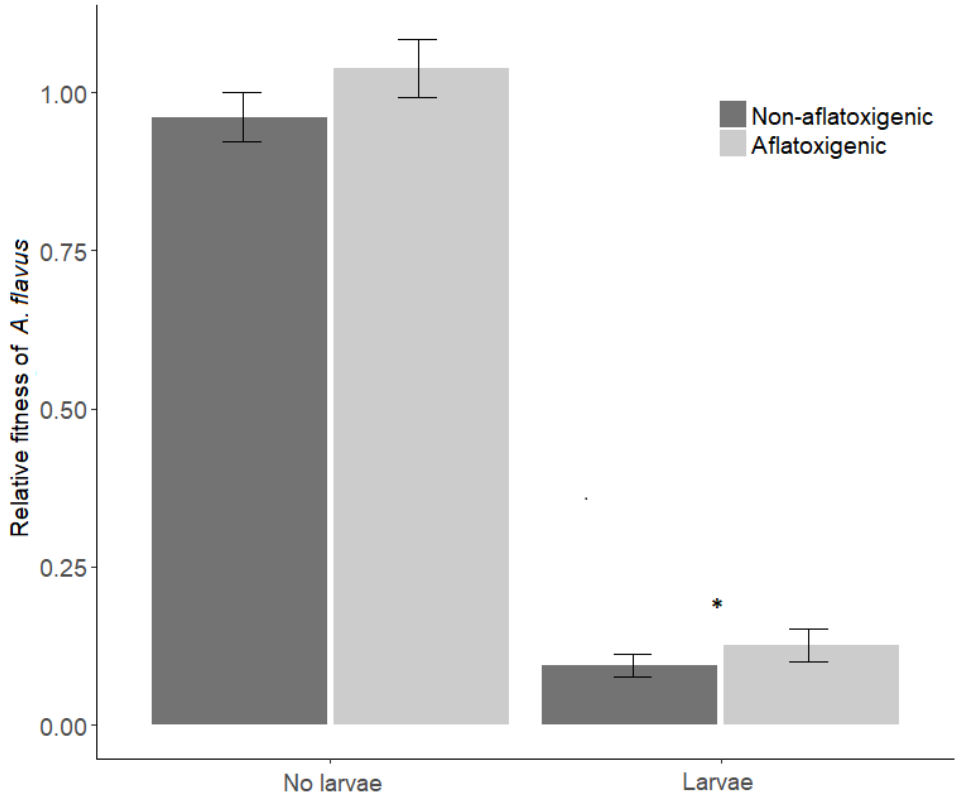

Figure 4. Average fitness of nine toxigenic and nine nontoxigenic isolates of *Aspergillus flavus* with and without competition from *Drosophila* larvae. Fitness was estimated by qPCR. Each isolate was replicated three times in each treatment (*N*=108). Error bars represent ±SE. When flies were added, fitness was slightly higher for toxigenic isolates than for non-toxigenic isolates (*P*=0.033 for one-sided hypothesis test that toxigenic isolates have greater fitness than nontoxigenic, indicated with '*').

Experiment 4: Effect of physical damage and feeding by *Drosophila* on aflatoxin production:

The type of physical damage that *A. flavus* experienced had a significant impact on the production of aflatoxin ($P<0.0001$). The simulated damage treatment produced an average of 5877 ppb aflatoxin, which was significantly higher than the average for larva treatment (3232 ppb, $P=0.0006$) and the average for the no-larva treatment (2121 ppb $P<0.0001$) (figure 5). The larva treatment also differed significantly from the no-larva treatment ($P=0.0065$). Aflatoxin production was not predicted by fungal fitness when used as a covariate ($P=0.4556$).

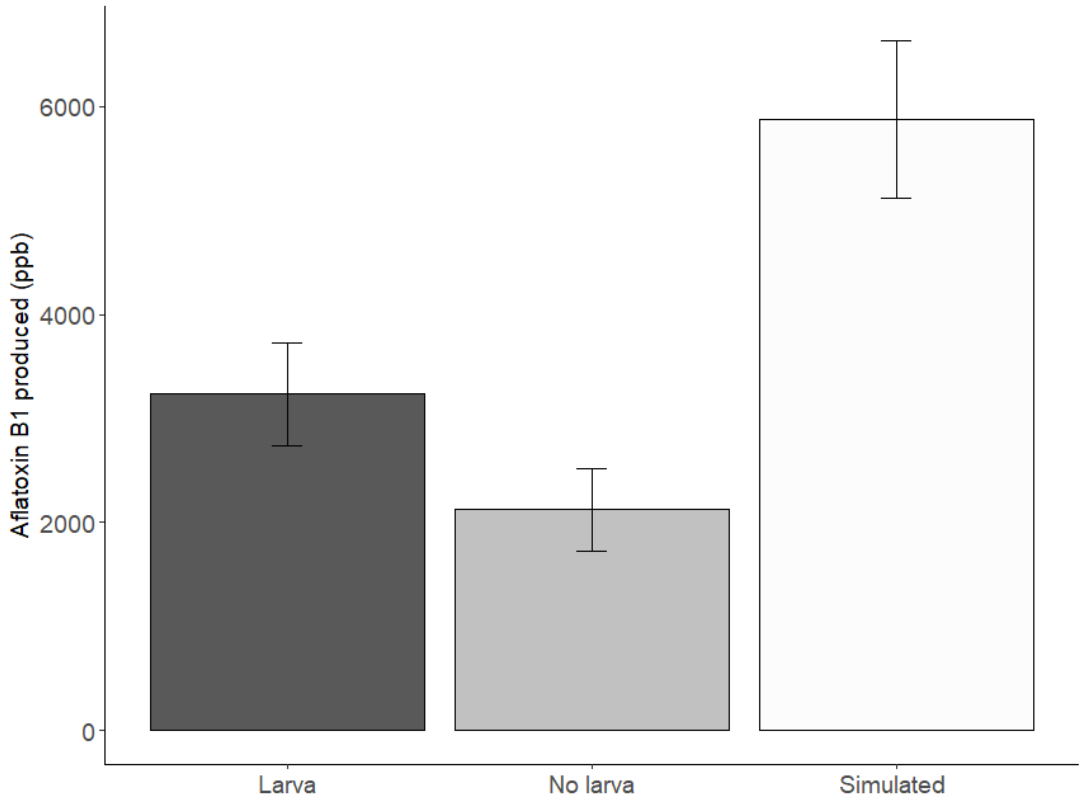

Figure 5. Average aflatoxin production of 12 toxigenic field isolates of *Aspergillus flavus* subjected to damage from a larva, no damage (no-larva), or simulated damage using a toothpick over the course of 72 h. Each isolate was replicated three times within each treatment (N=108). Error bars represent ±SE. Differences between all pairwise comparisons of treatments were highly significant (P<0.001)

Discussion:

We found that the addition of aflatoxin to *Drosophila* culture medium greatly reduced the fitness of *D. melanogaster* (figure 1). *Drosophila* larval mortality was greater in the presence of aflatoxigenic *A. flavus* isolates than in the presence of non-toxigenic isolates when aflatoxin was not added (figure 3). Reciprocally, when aflatoxin was added to the medium in the presence of larvae, fungal fitness increased linearly as aflatoxin concentration increased (figure 2). However, the addition of aflatoxin had no effect on fungal fitness in the absence of *Drosophila* larvae. Furthermore, toxigenic isolates had slightly higher fitness in the presence of larvae, but not in their absence (figure 4). Constitutive toxin production of natural toxigenic isolates *A. flavus* ranged from 8 ppb to 7000 ppb in our experiment, and there was a consistent induction of greater aflatoxin production when a larva interacted with the fungus or when physical damage to the fungus was simulated using a toothpick (figure 5). These results suggest that *Drosophila* larvae reduce fungal fitness, presumably by consuming the fungus or through resource competition, and that production of aflatoxin is a defense against the insect. The increase in fungal fitness associated with aflatoxin in the presence of insects, but not in their absence, is consistent with the hypothesis that aflatoxin is selected for through an interaction between fungi and insects. We assume that a cost of aflatoxin production in the absence of susceptible insects will favor non-toxigenic

isolates. Together these forces could maintain balancing selection for aflatoxin production.

Our initial measures of fly fitness in the absence of fungus on aflatoxin-containing medium (figure 1) do not provide evidence that aflatoxin may provide a selective advantage to the fungus, but instead show the potential toxicity of aflatoxin to *Drosophila* in our experimental system. Many studies have documented the variation in insect susceptibility to aflatoxin (Racovitza 1969, Niu, Wen et al. 2008, Rohlfs and Obmann 2009, Staaden, Milcu et al. 2010). Kroymann et al. (2003) suggest that comparable variation in the susceptibility of herbivores to glucosinolates produced by *Arabidopsis* could drive balancing selection evident in a glucosinolate biosynthesis gene. Similarly, we suggest that balancing selection proposed to operate on the aflatoxin gene cluster ((Carbone, Jakobek et al. 2007), Moore et al. 2009) could be driven by similar variation in insect susceptibility and relative abundance of associated insect species. For example, aflatoxin affects *Drosophila* but not maize weevils (*Sitophilis zeamais*). The addition of aflatoxin to medium acts to decrease *Drosophila* fitness while increasing fungal fitness (figures 3 and 2, respectively), and we expect the same to be true of other competitors and fungivorous insects. However, when we attempted experiments similar to those conducted in this study using maize weevils, we found no increase in mortality even when food contained 30,000 ppb aflatoxin (unpublished data). If the diversity and sensitivity to aflatoxin in insects associated with *A. flavus* vary over time or space, polymorphism for aflatoxin production could be adaptively maintained. Consistent with this hypothesis, Wicklow et al. (1994) speculated that geographic distributions seem to favor aflatoxigenic individuals in

warmer, lower latitudes (Manabe and Tsuruta 1978, Horn and Dorner 1999) that may coincide with the increased threat of fungivory from insects.

The biosynthesis of aflatoxin is energetically costly (Payne and Brown 1998, Calvo, Wilson et al. 2002, Yabe and Nakajima 2004). Given finite resources, the allocation cost of diverting energetic resources to defense has been demonstrated in several systems (Strauss, Rudgers et al. 2002). Thus, the allocation of resources to the production of aflatoxin when insects are present could create a fitness cost to *A. flavus*, ultimately driving balancing selection for the toxin. In our experiment, a cost of aflatoxin production could have manifested as a disproportionate benefit to non-toxigenic isolates interacting with insects when aflatoxin is added to the medium, but this is not what we observed (figure 2). We do not believe this contradicts the hypothesis that aflatoxin production is costly to fitness, however, since the cost of secondary metabolites is often measurable only in competitive, high stress, low resource situations (Lankau, Wheeler et al. 2011) and the cost of aflatoxin production could be hidden by the nutrient-rich synthetic medium we used for our experiments. A more competitive experimental setup was not feasible in our study due to the potential for "cheating" (discussed below). Our experimental medium is more nutrient-dense than most soils that are commonly thought to be the natural habitat of *A. flavus*. However, Wicklow et al. (1994) described Aspergilli in general as colonizing substrates in and on the soil, with little growth through the soil itself (similar to *Penicillium* growth pattern (Sewell 1959)). Our medium is a reasonable proxy for agricultural products like maize kernels that may fall to the soil surface but may not be for less nutrient-rich substrates, e.g., corn cobs.

When resources are scarce, plasticity in gene expression of putative defensive compounds has been suggested as a way of conserving energetic resources. In plant-herbivore systems this is often referred to as induced resistance. Demonstrating a benefit of induced resistance is difficult (reviewed by Heil and Baldwin 2002), partially because the range of ecological trade-offs may be offset by inducibility and the difficulty in establishing that experimentally measured costs definitively operate in the field (Agrawal 1998). Recently, evidence has been mounting that fungi are capable of induced resistance. Fungivory has been shown to increase secondary-metabolite gene expression and sexual spore formation in *A. nidulans* with a concurrent decrease in insect fitness (Döll, Chatterjee et al. 2013, Ortiz, Trienens et al. 2013). This result is consistent with our demonstration of increased aflatoxin production in the presence of insects (figure 5). However, our finding that physical damage increased aflatoxin production contrasts with studies that suggest acquired resistance is insect-mediated, not purely physical (Döll, Chatterjee et al. 2013, Ortiz, Trienens et al. 2013). The discordance is difficult to interpret in part because these studies used different modes of damage from each other and from our study. Furthermore, simulated herbivory often gives different responses from natural herbivory on plants (Baldwin 1990, Strauss and Agrawal 1999). Finally, cellular damage caused by physical maceration is known to cause the release of reactive oxygen species (Hernández-Oñate and Herrera-Estrella 2015), which have been associated with increased aflatoxin production (Jayashree and Subramanyam 2000, Reverberi, Fabbri et al. 2005). It is thus not possible to determine whether the alignment of increased aflatoxin production in both larva and simulated damage treatments is merely a coincidental effect of resulting

cellular conditions (i.e., a "spandrel" in Gould and Lewontin's (1979) analogy) or evidence of an adaptive response to fungal grazing. We speculate that these options are not necessarily mutually exclusive: fungi could have evolved to upregulate secondary metabolite pathways, recognizing oxidative stress response as evidence of fungivory. This sort of physical damage to a sessile microorganism may be most likely in the presence of insects. Increased aflatoxin production in the presence of *Drosophila*, regardless of mechanism, is consistent with an adaptive role for the toxin as a resistance trait to insects.

Although we have provided evidence that aflatoxin production benefits *A. flavus* when it is subject to fungivory, our results are also consistent with Janzen's (1977) hypothesis that aflatoxin mediates interference competition. *A. flavus* grows in and on nutrient-rich substrates such as seeds, potentially in competition with insects. During competitive interactions, insects may also engage in fungivory, making delineations between fungivory and competition difficult. In the context of this insect/fungus interaction, we posit that aflatoxin accumulated in fungal tissues (Wicklow and Shotwell 1982) inhibits fungivory, whereas aflatoxin secreted from the fungus into the substrate (Chanda, Roze et al. 2009, Chanda, Roze et al. 2010) benefits the fungus through interference competition. The two processes, however are not mutually exclusive and may interact in important ways that our experiments cannot separate. While we observed direct fungivory of *Drosophila* on *A. flavus* tissue (see video in Supplemental Materials), larvae also fed less on medium with higher aflatoxin concentrations. Given the short duration of our experiments, overall effects of

aflatoxin production by *A. flavus* in conditions without additional toxin supplementation may be due to accumulation in fungal cells before secretion. Addition of aflatoxin to growth medium has allowed us to demonstrate that both toxigenic and non-toxigenic isolates benefit from the toxin because of interference competition (figure 2). These aflatoxin levels are commonly encountered in agricultural commodities (Dorner, Cole et al. 1998, Lewis, Onsongo et al. 2005, Kumar, Basu et al. 2008) . The fact that both toxigenic and non-toxigenic *A. flavus* were able to benefit from the addition of aflatoxin to the medium raises the possibility that non-toxigenic individuals co-occurring with toxigenic individuals could also benefit from aflatoxin secreted by toxigenic individuals in the presence of insects. Similar cheating dynamics are known to maintain polymorphisms in some microbial systems. For example, genotypes of *Pseudomonas aeruginosa* that do not produce acyl-homoserine lactone (a quorum-sensing signal) realize a growth benefit when they co-occur with wild-type genotypes (Sandoz, Mitzimberg et al. 2007). The authors of that work suggested that a likely benefit was realized from not incurring the metabolic cost of producing the compound. While balancing selection for aflatoxin may benefit the fungus when susceptible insects are competing for resources, we cannot explicitly reject the hypothesis that cheating may also maintain this polymorphism.

Our results are the first clear evidence of a fitness advantage conferred to *A. flavus* by aflatoxin when interacting with insects. Our results are consistent with the hypothesis that insects drive balancing selection for aflatoxin production through interference competition and resistance to fungivory, and that the two may be inextricably linked. We present evidence that aflatoxin production is favored in the presence of insects and

may thus act as a driver of balancing selection, which does not preclude the toxin from having additional disparate functions including in interactions with ubiquitous soil microbes. The effect of aflatoxin on fungal fitness in a soil ecosystem remains an important area of study that may help elucidate costs of aflatoxin production as soils are often nutrient-poor. In addition to clarifying a long-standing hypothesis elaborated by Janzen (1977) on how balancing selection for aflatoxin may be maintained, our results emphasize the potential of non-toxigenic isolates to benefit from their toxigenic counterparts if co-occurring on a nutrient source. Instead of balancing selection being driven by the presence or absence of insects or other competitors, intraspecific cheating dynamics by non-toxigenic isolates is an alternative hypothesis that remains to be tested to explain balancing selection for aflatoxin production.

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CHAPTER 2

Fitness cost of aflatoxin production in *Aspergillus flavus* when competing with soil microbes could maintain balancing selection

Milton T. Drott[1], Tracy Debenport[1], Steven A. Higgins[2], Daniel H. Buckley[2], Michael G. Milgroom[1]

1. School of Integrative Plant Science, Plant Pathology and Plant-Microbe Biology Section, Cornell University, Ithaca, NY 14853
2. School of Integrative Plant Science, Soil and Crop Sciences Section, Cornell University, Ithaca, NY 14853

Key Words:

Balancing selection, aflatoxin, soil microbes, allocation cost, secondary metabolism, mycotoxins

Abstract:

Selective forces that maintain the polymorphism for aflatoxigenic and non-aflatoxigenic individuals of *Aspergillus flavus* are largely unknown. As soils are widely considered the natural habitat of *A. flavus*, we hypothesized that aflatoxin production would confer a fitness advantage in the soil environment. To test this hypothesis, we used *A. flavus* DNA quantified by qPCR as a proxy for fitness of aflatoxigenic and non-aflatoxigenic field isolates grown in soil microcosms. Contrary to predictions, aflatoxigenic isolates had significantly lower fitness than non-aflatoxigenic isolates across three temperatures (25, 37, 42 °C) in natural soils. When we combined data from three experiments in natural soil at 37 °C, aflatoxigenic isolates had significantly lower fitness than non-aflatoxigenic isolates. The addition of aflatoxin to soils (500 ng/g) had no effect on the growth of *A. flavus*. Amplicon sequencing showed that neither the aflatoxin-producing ability of the fungus, nor the addition of aflatoxin had a significant effect on the composition of fungal or bacterial communities in soil. We argue that the fitness disadvantage of aflatoxigenic isolates is most likely explained by the metabolic cost of producing aflatoxin. Coupled with a previous report of a selective advantage of aflatoxin production in the presence of insects, this fitness cost in soil could provide a mechanism by which balancing selection maintains the polymorphism in aflatoxin production in *A. flavus*.

Introduction:

Although there is a large body of work *in vitro* focused on the role of microbial secondary metabolites, the ecological function of these compounds remains poorly understood. Even the ecological role of antibiotics is being questioned because soils, the natural environment for many of the microbes that produce antibiotics, are now commonly thought to contain sub-inhibitory concentrations of these compounds (Allen, Donato et al. 2010, Bellemain, Davey et al. 2013). Beyond antibiotics, few microbial secondary metabolites have received as much attention as aflatoxin, which is produced by the fungi *Aspergillus flavus, A. parasiticus,* and a few other closely related *Aspergillus* species. Aflatoxin is an extremely potent hepatotoxin that causes acute toxicosis, cancer, immune suppression, and stunted growth in children (Williams, Phillips et al. 2004, Wild 2007, Liu and Wu 2010, Wild and Gong 2010, 2013). Contamination of corn, peanuts, cotton, and tree nuts with this toxin is estimated to cost hundreds of millions of dollars annually in the United States alone (Robens and Cardwell 2005). However, not all strains of *A. flavus* produce aflatoxin. Extensive field sampling of *A. flavus* in the US found that 29% of all isolates did not produce aflatoxin (Horn and Dorner 1999). Worldwide, both chemotypes, fungal isolates that produce or do not produce aflatoxin (aflatoxigenic and non-aflatoxigenic, respectively), are often found in soil in the same field (Horn 2003). Nucleotide sequence analysis of 21 regions in the aflatoxin gene cluster in *A. flavus* and *A. parasiticus* confirmed that polymorphism for aflatoxin production is maintained by balancing selection (Carbone, Jakobek et al. 2007, Moore, Singh et al. 2009). As with antibiotics, however, attempts to understand the forces selecting for– and against–

aflatoxin production have been done in *in vitro* laboratory assays that are not representative of conditions the fungus would encounter in its natural habitat.

To maintain the polymorphism for aflatoxin production by balancing selection, aflatoxigenic individuals must be favored under some conditions while non-aflatoxigenic individuals are favored under others. Without selection favoring each chemotype under different conditions, one of the chemotypes should become fixed. Janzen (1977) hypothesized that aflatoxin production is favored in the presence of insects, birds, mammals or soil microbes through interference competition. Under this hypothesis, the toxic effects of aflatoxin produced in nutrient-rich substrates like seeds increases the fitness of fungi producing them by deterring competitors. Recently, Drott et al. (Drott, Lazzaro et al. 2017) provided evidence that aflatoxin production increased the fitness of *A. flavus* in the presence of some insects, but not in their absence. They speculated, that *A. flavus* might incur a fitness cost associated with the biosynthesis of aflatoxin in the absence of insects because it is energetically costly to produce (Payne and Brown 1998, Calvo, Wilson et al. 2002, Yabe and Nakajima 2004). However, if there were a cost, it may have been masked in the experiments of Drott et al. (Drott, Lazzaro et al. 2017) because nutrient-rich media was used or the differences may have been too small to detect experimentally. Nonetheless, the fitness advantage observed in the presence of insects, together with the proposed cost of production, could drive balancing selection as a function of the presence of insects and their susceptibility to aflatoxin. This explanation for the balancing selection acting on aflatoxin production under these conditions does not, however, preclude the toxin from having benefits (or costs) in other environments.

Soil is widely considered the natural habitat of *A. flavus* and is thus a likely environment for aflatoxin production to benefit the fungus through interference competition with microbes. However, little is known about the ecology of the fungus or the role of aflatoxin in soil environments (Orum, Bigelow et al. 1997, Geiser, Dorner et al. 2000, Horn 2003, Jaime-Garcia and Cotty 2004). *A. flavus* actively colonizes organic matter in or on soil with little growth through the soil itself (Wicklow, Dowd et al. 1994). Both aflatoxigenic and non-aflatoxigenic chemotypes of *A. flavus* are commonly isolated from agricultural soils (Horn and Dorner 1999, Horn 2003). Warmer, lower latitudes seem to favor aflatoxigenic strains (Manabe, Tsuruta et al. 1976). Wicklow (1994) speculated that the higher frequencies of aflatoxigenic isolates in soil at these latitudes may be correlated with greater densities of soil insects associated with a relatively warm climate. However, there is little or no evidence supporting this speculation. We hypothesize that microbial communities may also select for aflatoxigenic individuals in warmer soils. Under the interference competition hypothesis, in regions where aflatoxigenic isolates are more common, *A. flavus* would suppress competition with soil microbes by producing aflatoxin. Consistent with this hypothesis, there appears to be a relationship between aflatoxin production and soil microbes; aflatoxin production is induced by several soil bacteria and yeast *in vitro* (Wicklow, CW et al. 1980, Cuero, Smith et al. 1987). Moreover, expression of aflatoxin biosynthetic genes in *A. flavus* and aflatoxin *per se* have been observed in soil (Accinelli, Abbas et al. 2008). Little is known, however, about the impact of aflatoxin production on the fitness of *A. flavus* and the composition of the microbial community in soil.

Support for the hypothesis that aflatoxin mediates interference competition with soil microbes is mixed. Aflatoxin has been reported as having limited impact on soil microbes *in vitro*, having little to no effect on growth even at concentrations well above those observed in contaminated agricultural commodities (Burmeister and Hesseltine 1966, Arai, Ito et al. 1967). In contrast, however, Angle and Wagner (1981) showed that at high concentrations (10,000 ppb), aflatoxin B_1 reduced the number of viable fungal and bacterial propagules *in vitro*. Such decreases in microbial population density could be interpreted as support for interference competition mediated by aflatoxin. However, that study did not assess the fitness of *A. flavus* or identify specific, ecologically relevant, microbial species. Though methods are now available to estimate fitness and assess the composition of microbial communities in soil, we still lack information on specific microbes interacting with *A. flavus* or being inhibited by aflatoxin. Several studies have demonstrated the mutual inhibition of *A. flavus* and various *Bacillus* species (Burmeister and Hesseltine 1966, Cuero, Smith et al. 1987, Faraj, Smith et al. 1993). Some of these same *Bacillus* species are more sensitive to the antibiotic effects of aflatoxin than most other bacteria (Burmeister and Hesseltine 1966, Cuero, Smith et al. 1987, Faraj, Smith et al. 1993). Interestingly, both *A. flavus* and *Bacillus* populations are largest in fields with persistent drought and high-temperature stress environments (>35 °C) (10), potentially providing an environment for direct competition between these microbes. These studies, done either in the absence of the fungus and/or *in vitro*, leave open the possibility that aflatoxin acts through interference competition to confer a fitness advantage when competing with soil microbes, much as it does in the presence of some insects (13).

We sought to determine whether interference competition with soil microorganisms by the production of aflatoxin confers a fitness advantage to *A. flavus* and thus partially explains balancing selection for aflatoxin production. Specifically we addressed the following questions: 1) Does the production, or addition, of aflatoxin increase the fitness of *A. flavus* in soil? 2) Does the production of aflatoxin by *A. flavus*, or presence of aflatoxin in soil affect microbial community composition? We used culture-independent methods to determine the effect of aflatoxin on the fitness of *A. flavus* during its interaction with soil microbes in field-soil microcosms. We compared naturally occurring aflatoxigenic and non-aflatoxigenic strains of *A. flavus*, in sterile and non-sterile (natural) soil to examine the effects of soil microbes on the fitness of *A. flavus*.

Results:

Effects of aflatoxin on fitness of *A. flavus* in sterile and natural soils

Experiment 1: We observed a significant interaction of soil sterility and temperature with respect to their effects on the fitness of *A. flavus* (ANOVA, $F_{2,67}$=12.8, P<0.0001, Table S1). This interaction is evident in the difference in average fitness between sterile and natural soils, which is smaller at 37 °C and 42 °C (regardless of chemotype) and larger at 25 °C (Fig. 1). Although we did not observe any difference between aflatoxigenic and non-aflatoxigenic isolates at any specific temperature (Tukey post-hoc, P>0.616), there was a significant interaction between chemotype and the soil sterility independent of temperature (ANOVA, $F_{1,67}$=4.2, P=0.043, Table S1). This interaction manifests with aflatoxigenic isolates having lower fitness than non-aflatoxigenic isolates in natural soils, but not in sterile soils (Fig. 1). While there was

no difference in fitness of both choemotypes between sterile soils incubated at 25 and 37 °C (Tukey post-hoc, P>0.928), fitness was reduced 93% in natural soils incubated at 25 °C when compared to fitness in natural soils at 37 °C (Tukey post-hoc, P<0.0001).

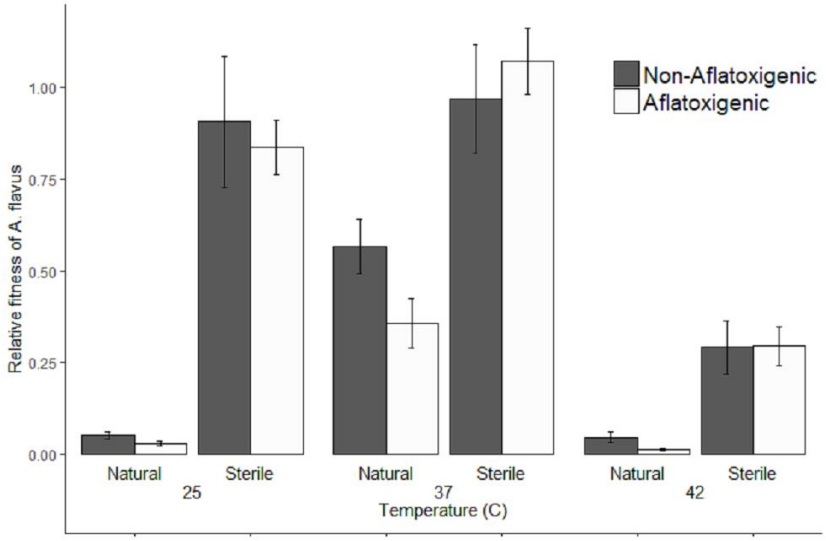

Fig. 1. Mean relative fitness of aflatoxigenic (N=4) and non-aflatoxigenic (N=3) isolates of *Aspergillus flavus* in natural and sterile field soils at three temperatures (Experiment 1). Fitness of *A. flavus* was estimated by qPCR to quantify DNA relative to a standard curve. Each treatment was replicated twice and incubated for 4 days. Error bars represent ± SE. Aflatoxigenic isolates had lower fitness than non-aflatoxigenic isolates in natural soils but not in sterile as indicated by the significant interaction between toxin producing ability and soil sterility (P=0.043)

Experiment 2: Because the fitness of *A. flavus* in natural soils was observed to be greatest at 37 °C we further investigated the effect of aflatoxin production and aflatoxin added to the soil (500 ng/g soil (ppb)) on *A. flavus* fitness at this temperature. Again we observed that aflatoxigenic isolates had significantly lower fitness than non-aflatoxigenic isolates in natural soils (ANOVA, $F_{1,9}$=5.2, P=0.049, Table S2), but not in sterile soils (ANOVA, $F_{1,9}$=1.4, P=0.271, Table S3) (Fig. 2). The addition of aflatoxin had no effect on the fitness of either chemotype in natural (ANOVA,

$F_{1,31}$=2.5, *P*=0.124, Table S2) or sterile soil (ANOVA, $F_{1,31}$=0.66, *P*=0.8, Table S3). The interaction between added aflatoxin and chemotype was not significant in either natural (ANOVA, $F_{1,31}$=0.9, *P*=0.349, Table S2) or sterile soils (ANOVA, $F_{1,31}$=0.006, *P*=0.937, Table S3).

Experiment 3: Since Experiments 1 and 2 unexpectedly showed that aflatoxigenic isolates had lower fitness than non-aflatoxigenic isolates in natural soil, we decided to estimate the fitness, in natural soil, of an additional sample of 20 aflatoxigenic and seven non-aflatoxigenic *A. flavus* field isolates. In this experiment, the mean fitness of aflatoxigenic and non-aflatoxigenic isolates was not significantly different (ANOVA, $F_{1,25}$=0.13, *P* = 0.72, Table S4) (Fig. 3). However, when *A. flavus* fitness data (from natural soil incubated at 37 °C without aflatoxin added) were combined from Experiments 1-3, aflatoxigenic isolates had significantly lower fitness than non-aflatoxigenic isolates (ANOVA, $F_{1,39.9}$=4.1, *P*=0.050, Table S5). In this combined analysis, there was a significant effect of the 'experiment' blocking variable on fitness (ANOVA, $F_{2,82.7}$=6.6, *P*=0.002, Table S5). This effect of experiment on fitness, however, did not interact with the effect of chemotype (ANOVA, $F_{2,82.7}$=1.4, *P*=0.242, Table S5).

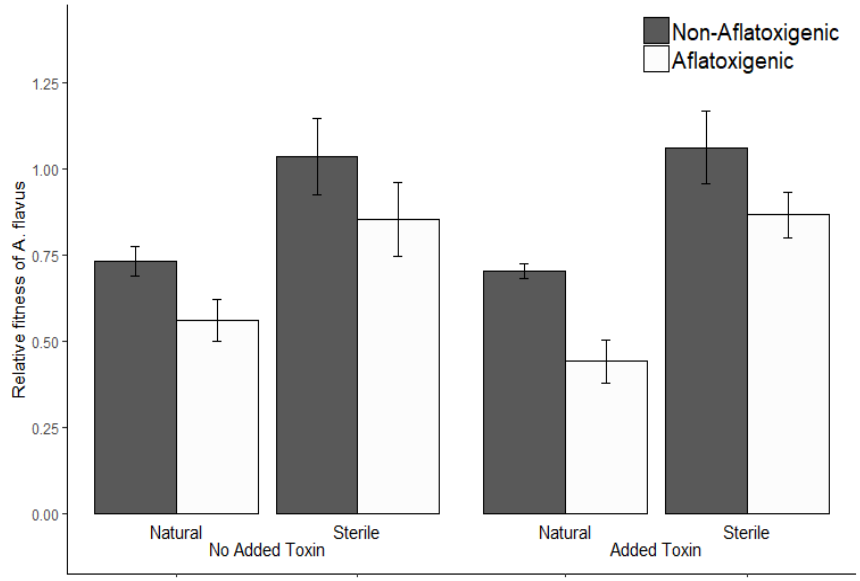

Fig. 2. Mean relative fitness of aflatoxigenic (*N*=7) and non-aflatoxigenic (*N*=4) isolates of *Aspergillus flavus* in natural and sterile field soils with and without 500 ng/g soil (ppb) aflatoxin added. Fitness of *A. flavus* was estimated by qPCR relative to a standard curve. Each treatment was replicated twice and incubated for four days. Error bars represent ± SE. Fitness of aflatoxigenic isolates was significantly lower than non-aflatoxigenic isolates in natural soils, (*P*=0.049) but not in sterile soils (*P*=0.271).

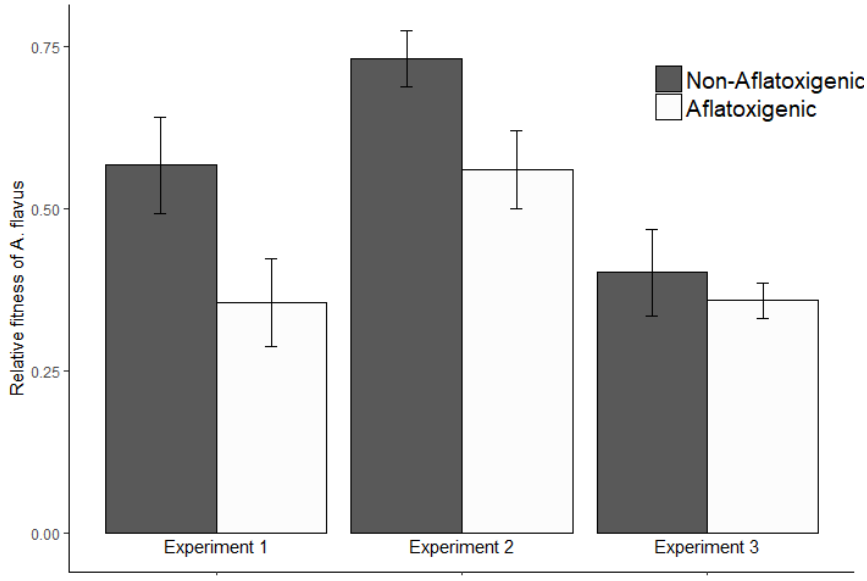

Fig. 3. Mean relative fitness of aflatoxigenic and non-aflatoxigenic fungal isolates from Experiments 1, 2 and 3, all in the natural soil at 37 °C. These comparable data from the three experiments contained a total of 27 aflatoxigenic and 11 non-

aflatoxigenic clone-corrected isolates respectively. Fitness of *A. flavus* was estimated by qPCR relative to standard curves. Each microcosm was replicated twice and incubated for four days (N=90). Error bars represent ± SE. Fitness of aflatoxigenic isolates was significantly lower than non-aflatoxigenic isolates across all experiments (P=0.05).

Effects of aflatoxin on soil microbial communities

Amplicon sequencing of fungal ITS and bacterial SSU rRNA genes from natural soils in Experiment 2 revealed that the alpha diversity of the fungal community, as measured by the Shannon index, was unaffected by either the addition of aflatoxin to soil (1.6 ± 0.37 and 1.58 ± 0.49 with and without aflatoxin, respectively; mean ± s.d.), or the chemotype of isolates (1.53 ± 0.48 and 1.67 ± 0.34 for aflatoxigenic and non-aflatoxigenic, respectively) (ANOVA, $F_{1,44}$=0.017, P=0.898, and $F=_{1,44}$=0.033, P=0.259, Table S6, respectively). Similarly, bacterial community alpha diversity was indistinguishable for both aflatoxin amendments (5.98 ± 0.09 and 5.99 ± 0.11 with and without added aflatoxin, respectively) and chemotype (5.98 ± 0.09 and 5.99 ± 0.11 for aflatoxigenic and non-aflatoxigenic isolates, respectively) (ANOVA, $F_{1,44}$=1.15, P=0.289 and, $F_{1,44}$=2.55 P=0.118, Table S7, respectively). In addition, fungal beta diversity was unaffected by chemotype (PERMANOVA, R^2= 0.03, P= 0.144, Table S8), though there was a small but significant effect of chemotype on bacterial beta diversity (PERMANOVA, R^2 = 0.03, P=0.048, Table S9) (Fig. 4). Analysis of all bacterial OTUs, including all OTUs annotated as *Bacillus* spp., indicated no change in the relative abundance of individual taxa among treatments (Fig. S1-S4). We thus conclude that neither aflatoxin production nor the addition of aflatoxins to soil had a meaningful effect on the fungal or bacterial communities in natural soil.

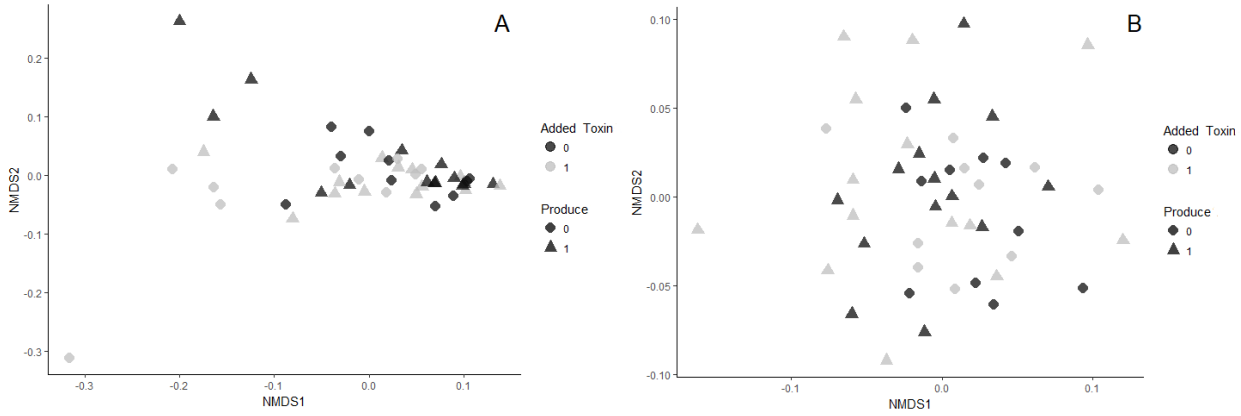

Fig. 4. Nonmetric multidimensional scaling (NMDS) analysis representing similarity in fungal community (A) and bacterial community (B) structure in soil microcosms incubated with (black) and without (gray) the addition of aflatoxin after inoculation with non-aflatoxigenic (circles) or aflatoxigenic (triangles) isolates of *A. flavus*. Results are from amplicon sequencing of DNA samples from natural soils in Experiment 2.

Discussion:

Balancing selection for aflatoxin production is evident from the persistent polymorphism in the ability to produce aflatoxin among isolates of *A. flavus* found together in the same field (Horn 2003), and in molecular signatures in the aflatoxin gene cluster (Carbone, Jakobek et al. 2007, Moore, Singh et al. 2009). Recently Drott et al. (Drott, Lazzaro et al. 2017) provided experimental support for the hypothesis of Janzen (Janzen 1977), which is that aflatoxigenic isolates of *A. flavus* have a fitness advantage over non-aflatoxigenic isolates because of interference competition with insects. Because soil is often reported as the natural habitat of *A. flavus* (Orum, Bigelow et al. 1997, Geiser, Dorner et al. 2000, Horn 2003, Jaime-Garcia and Cotty 2004, Accinelli, Abbas et al. 2008), we tested a corollary to Janzen's (Janzen 1977) hypothesis that aflatoxin production would also benefit aflatoxigenic isolates through interference competition with soil microbes. Contrary to expectations, we

demonstrated that aflatoxigenic isolates have lower fitness than non-aflatoxigenic isolates in the presence of microbes, but not in sterile soil. We found that the microbial communities in natural soils markedly reduced the absolute fitness of *A. flavus* compared to fitness in sterile soils. However, neither the addition of aflatoxin nor the aflatoxin-producing ability of the fungus (chemotype) had an effect on overall microbial community composition or on the relative abundance of specific taxa between treatments. Therefore, given the lack of effect of aflatoxin on microbial communities, we attribute differences in fitness to a metabolic cost of producing aflatoxin in natural soil. We speculate that this fitness cost can be detected in natural soil because of low-nutrient conditions caused by competition with microbes, whereas the cost of aflatoxin production may be smaller and not detectable when conditions are more favorable. Thus, in natural soil, in the presence of microbes, aflatoxigenic individuals may be selected against relative to non-aflatoxigenic individuals. Coupled with the findings of Drott et al. (2017), our study suggests that competition with insects selects for aflatoxigenic isolates, whereas competition with soil microbes selects for non-aflatoxigenic isolates. Together, these selective forces may maintain the polymorphism observed for aflatoxin production in *A. flavus*.

The potential role of aflatoxin as an agent of interference competition in the soil ecology of *A. flavus* has previously been questioned because aflatoxin is quickly degraded in soil (Cotty, Bayman et al. 1994). However, in pure culture and in soil, large amounts of aflatoxin inhibit the growth of some bacteria (Burmeister and Hesseltine 1966, Arai, Ito et al. 1967, Angle and Wagner 1981) and fungi (Angle and Wagner 1981). Although we did not quantify microbial populations overall, we did

not find any effect of the production of aflatoxin by *A. flavus* or of the addition of pure aflatoxin on the soil microbial community composition. It is possible that our observation that aflatoxin had little impact compared to previous studies was due to our use of lower aflatoxin concentrations, resembling those found in soil. Given past observation of the expression of aflatoxin biosynthetic genes in soil (Accinelli, Abbas et al. 2008) we assume that with the low *A. flavus* biomass we observed in soil, aflatoxigenic isolates produce aflatoxin at rates that do not typically keep up with the degradation of aflatoxin we observed, even under sterile conditions (Fig. S5). Such degradation may prevent the accumulation of measurable amounts of toxin in soils, perhaps minimizing a role for aflatoxin in interference competition with soil microbes. The shorter duration of our experiments (4 days), relative to previous experiments (70 days) may also explain, in part, why we did not see changes in microbial communities. *A. flavus* is relatively fast-growing, and quickly colonizes organic matter that may fall to the soil surface while not growing into the soil itself (Wicklow, Dowd et al. 1994). Given this life history strategy, we believe that the shorter timeline of our study is representative of the ecology of *A. flavus*, although aflatoxin may impact soil microbial community structure over larger time scales not examined here.

While we did not observe any effect of aflatoxin on the composition of soil microbial communities, it is clear that these communities greatly affect the growth of the fungus. The fitness of *A. flavus* in sterile soils was unchanged between 25 °C and 37 °C; however, in natural soils, fitness was an order of magnitude lower at the cooler temperature (Fig. 1). This finding is consistent with reports of suppressive soils on some plant pathogenic fungi, where competition with other soil microbes decreases

the pathogen's fitness (reviewed by (Weller, Raaijmakers et al. 2002)). As we observed, the suppressiveness of soil microbes can be mediated by abiotic factors like temperature (reviewed by (Burpee 1990)). In fact, Henry (Henry 1932) speculated that the higher incidence of 'take-all' disease of wheat (caused by the fungus, *Gaeumannomyces graminis* var. *tritici*) at more northern latitudes and during colder parts of the season, may partially be explained by the temperature-dependence of the suppressive effect, which is diminished in colder temperatures. Analogously, we speculate that lower population density of *A. flavus* at cooler latitudes (Manabe, Tsuruta et al. 1976, Manabe and Tsuruta 1978) may partially be explained by greater suppressive effects of soil microbes, as observed here. These results, however, do not elucidate any particular interaction between soil microbial communities and aflatoxin production by *A. flavus*. As the microbial suppression we observed appears to be independent of aflatoxin production, we speculate that the most likely explanation for the fitness cost we observed is the energetic cost of aflatoxin production.

Fitness costs associated with specific genes are notoriously difficult to demonstrate in many systems (Zhan and McDonald 2013). This is due to the complex ecological interactions and the 'noise' associated with measuring fitness under conditions representative of the field (Agrawal 1998). Even when fitness differences are large, it has been difficult sometimes to quantify fitness costs. For example, the fitness cost associated with producing the host-specific T-toxin by race T of *Cochliobolus heterostrophus* was thought to be relatively large because the frequency of race T decreased very rapidly when selection for it was removed by radical changes in host populations (Leonard 1977). However, attempts to demonstrate a fitness cost

experimentally, using isogenic lines, resulted in estimates that were not statistically significant between the two races (Leonard 1977).The fitness cost we observed for aflatoxin production thus is an important demonstration of the cost of secondary metabolite production in fungi. The effect is, however, small, as is evident from the lack of significant difference between the fitness of aflatoxigenic and non-aflatoxigenic isolates in sterile soil or even in natural soil in one experiment (Experiment 3). Yet this small difference in natural soils was significant when all experiments were analyzed together (Fig. 3). Our subjection of *A. flavus* to the nutrient-poor and highly competitive conditions of soils containing microbes may have created an environment stressful enough to show this cost in our experimental microcosms. As *A. flavus* does not readily reproduce sexually *in vitro*, it is not possible for us to construct isogenic lines that would verify that fitness costs we observed are caused by a metabolic cost of aflatoxin production.

Given the clonal nature of *A. flavus* (Horn 2003), the differences we saw in fitness of aflatoxigenic and non-aflatoxigenic field isolates in competition with soil microbes may be caused by linkage disequilibrium between the aflatoxin gene cluster and other genes. Aflatoxigenic isolates and non-aflatoxigenic isolates have been reported from separate clonal lineages (Horn, Greene et al. 1996, Horn, Moore et al. 2009). While it has recently been shown that *A. flavus* is capable of sexual reproduction *in vitro* on synthetic media (Horn, Moore et al. 2009), and on corn, no ascospores have been reported in nature (Horn, Sorensen et al. 2014). Despite the clonal nature of the fungus in agricultural populations (Horn 2003), balancing selection for aflatoxin production is ancient and predates speciation of *A. flavus* and

the closely related *Aspergillus parasiticus* (Carbone, Jakobek et al. 2007). Recombination has been observed within the aflatoxin gene clusters of both species (Carbone, Jakobek et al. 2007, Moore, Singh et al. 2009) and is likely responsible for the separation of the aflatoxin gene cluster from the neighboring cyclopiazonic acid gene cluster in some lineages (Moore, Singh et al. 2009). Aflatoxin-producing ability is thus linked to many genes within a clonal lineage, but not between lineages. While we cannot fully rule out the possible confounding effect of linkage disequilibrium, the isolates we used are a clone-corrected random sample of *A. flavus* populations in the United States and thus serve to explain the balancing selection observed in these populations.

To our knowledge, these results are the first evidence of a fitness cost of the production of aflatoxin in *A. flavus*. When coupled with previous findings that aflatoxin production benefits the fungus in the presence of insects (Drott, Lazzaro et al. 2017), this opposing selective force may explain the maintenance of chemotype polymorphisms (Horn and Dorner 1999) and signatures of balancing selection observed in the aflatoxin gene cluster (Moore, Singh et al. 2009). We speculate that when *A. flavus* competes with soil microbes for small pieces of organic matter (as used in this study), and in the absence of insects or other invertebrates, aflatoxin does not provide any fitness benefit and is costly to produce. This cost, however, may be negligible (or too small to detect) when nutrients are plentiful, as on laboratory media *in vitro* or in microcosms with sterile soil. Conversely, the cost of aflatoxin production may be outweighed by a fitness advantage when insects are present (Drott, Lazzaro et al. 2017). Although these studies together create a theoretical framework that explains

balancing selection, they do not preclude other fitness benefits or costs of aflatoxin production that have yet to be measured. Furthermore, it is unclear what impact, if any, the forces demonstrated in laboratory microcosms have in natural conditions. We suggest that current attempts at biological control of aflatoxin in agricultural fields may be a useful context in which the selective forces we have described are relevant. Biological control is effected by the competitive displacement of aflatoxigenic isolates by applying high densities of non-aflatoxigenic *A. flavus* propagules (Reviewed by Ehrlich (2014)). In particular our results suggest that selective pressures acting on aflatoxigenic and non-aflatoxigenic individuals are different. Differential fitness of *A. flavus* chemotypes in specific environments can be interpreted as differences in niche breadth, which is an important component of niche overlap (Levins 1968). Thus the competitive differences we have observed between *A. flavus* and soil microbes or insects indicate that the niches of aflatoxigenic and non-aflatoxigenic individuals do not completely overlap. Conversely the niches of individuals within a chemotype overlap completely in this respect. As competition increases with increased niche overlap (44), biocontrol applications may have a disproportionately greater effect on the diversity and prevalence of indigenous non-aflatoxigenic individuals than on aflatoxigenic ones. If such decreases in the diversity occur biocontrol may inadvertently diminish the ability of non-aflatoxigenic *A. flavus* populations to compete with aflatoxigenic populations, potentially affecting aflatoxin contamination of crops.

Methods:

Cultures of *Aspergillus flavus*:

Aspergillus flavus was isolated between 2013 and 2017 from independent soil samples from corn fields in Pennsylvania, North Carolina, and Florida, Texas, and Oklahoma (Table S10) using dilution-plating methods (Horn and Dorner 1999). Resulting colonies were streaked onto 25% Czapek Agar (CZA; 2% agar and 12.25 g Czapek medium (Difco Laboratories, Detroit, MI), which is 25% the recommended rate) and incubated for 16 h at 30 °C in the dark. For each colony, a single hyphal tip was transferred onto CZA to ensure the presence of only one genotype. Isolates were identified to the species level based on morphology (Horn and Dorner 1998). Identification was further confirmed against closely related species with similar morphology (e.g., *Aspergillus nomius*) by BLASTn (NCBI) analysis of β-tubulin sequences similar to methods described previously (Tam, Chen et al. 2014).
All isolates were cultured in yeast extract sucrose (YES) medium (Horn and Dorner 1999) and on Drosophila culture medium (DCM) (13) to determine aflatoxin chemotype by HPLC, as described previously (13). Cultures on DCM were mechanically damaged with a sterile tooth pick to stimulate greater production of aflatoxin (Drott, Lazzaro et al. 2017). Both of these assays were replicated twice for all isolates. Although *A. flavus* has the potential to produce a variety of mycotoxins, for example, cyclopiazonic acid (CPA), whose biosynthetic cluster is adjacent to the aflatoxin cluster (Horn, Moore et al. 2009), in this paper we refer to isolates and their chemotype as aflatoxigenic and non-aflatoxigenic based solely on their ability to produce aflatoxin B_1.

Experimental Soil Microcosms:

Soil used in microcosms was collected from the top 2 cm of an agricultural field in Ithaca, New York. Soils from this field are characterized as Langford Channery silt loam, with 2-8% slopes, eroded. Soil was air dried at room temperature for 4 d, passed through a 0.5 mm sieve to homogenize, and stored at 4 °C for no longer than 3 months before use. While several *Aspergillus* spp. were commonly observed in this field soil, repeated attempts to isolate and detect *A. flavus* by qPCR (described below) indicated that *A. flavus* was not present. Autoclaved soil (40 minute liquid cycle) was used in experiments where sterile soil was needed. We found no difference in the growth of *A. flavus* in microcosms containing autoclaved soils compared to γ-irradiated soils (Penn State Radiation Science and Engineering Center, University Park, PA).

Soil microcosms consisted of 5 g soil in 50-ml plastic screw-cap tubes. To each tube we added 0.3 g corn meal that was sieved to obtain particles between 0.25-0.5 mm in size. We conducted a blind study in which fungal isolates were assigned a random number independent of relevant meta-data until after analysis was completed. *A. flavus* inoculum was added to soil microcosms in 410 μl H_2O at 120 spores/μl (for a final concentration of ~10,000 spores/g soil) (Supplemental methods 1). This spore density is consistent with several studies that have quantified *A. flavus* in field soils (Abbas, Zablotowicz et al. 2004). Microcosms were incubated for 4-20 days depending on the experiment as indicated below.

Quantitative PCR (qPCR) for estimating fitness of *A. flavus* in soil microcosms:

After 4 days incubation, soil microcosms were homogenized by vortexing three times for 30 s each, with vigorous shaking by hand after each vortex. DNA was extracted

from 0.25 g of soil using MoBio Power Soil kit (MoBio Laboratories, Solana Beach, CA) following their DNeasy PowerSoil Protocol for Low Biomass Soil with RNase. We modified the protocol with the addition of three phenol:chloroform:isoamyl alcohol (24:8:1) extractions after the initial vortex, and the addition of a 600-µl 70% ethanol wash of the spin column immediately before elution of DNA.

We quantified DNA of *A. flavus* by qPCR as a proxy for fungal fitness using primers, reactions conditions, and methods previously described (Drott, Lazzaro et al. 2017). We used a genetic marker in the *omtA-1* gene in the aflatoxin biosynthetic cluster (13). Tests of several other PCR primer pairs were not specific enough to *A. flavus* to reliably quantify its biomass in field soil (Table S11). Use of *omtA-1* resulted in 11 non-aflatoxigenic isolates whose DNA could not be amplified with this marker, presumably because this gene has been deleted (Chang, Horn et al. 2005).

Standard curves were constructed by pooling three randomly selected experimental DNAs and creating a dilution series as described previously (Drott, Lazzaro et al. 2017). A subset of DNAs were also chosen at random to confirm qPCR efficiency in all experimental conditions. All efficiencies were between 90-100% with r^2 >0.99. Standard curves were identical for all plates within an experiment, but not between experiments.

<u>HPLC for quantifying aflatoxin from soil:</u>

Aflatoxin was extracted from total soil remaining after DNA extraction by adding 2.5 ml diH2O and 7.5 ml ethyl acetate to each microcosm tube (Accinelli, Abbas et al. 2008). The mixture was shaken overnight at 100 rpm on an orbital shaker (Lab-Line, Melrose Park, IL), centrifuged at 2500 × g for 10 min, and 6 ml of the organic layer

was reduced to dryness in a silanized tube under nitrogen stream. Aflatoxin was dissolved in 1 ml of 45% methanol and quantified by HPLC as described previously (Drott, Lazzaro et al. 2017) except for the use of a ZORBAX Eclipse XDB C18, 4.6 x 150-mm, 3.5 μm column (Agilent Technologies, Santa Clara, CA, USA) at a flow rate of 1 ml/min to improve detection. Yields, as tested by adding pure aflatoxin to soil in methanol, were ~40%. Reported ppb values have been corrected for yield. Tests of aflatoxin degradation in natural and sterile soils were also conducted (Supplemental methods 2, Fig. S5.).

Effects of aflatoxin on fitness of *A. flavus* in soils:

The effect of various soil conditions on fungal fitness were tested in three experiments:

Experiment 1: To test the effect of the interaction of temperature and presence of soil microbes on fitness of both chemotypes, four aflatoxigenic and three non-aflatoxigenic isolates were randomly assigned to separate microcosms with sterile or natural soils and incubated at 25, 37, and 42 °C for 4 days. Every isolate was replicated twice under each condition in a full factorial design. Additionally a tube with no added *A. flavus* was included at each condition as a control.

Experiment 2: To test the potential of added aflatoxins to affect fitness of *A. flavus*, microcosms with sterile or natural soil were inoculated with spores from one of 12 *A. flavus* isolates. Each of seven aflatoxigenic and five non-aflatoxigenic isolates were replicated twice with and without 500 ppb (ng/g soil) aflatoxin added. Resulting microcosms were incubated at 37 °C for 4 days. DNA samples from microcosms

containing natural soil in this experiment were later used in amplicon sequencing analysis.

Experiment 3: To further test patterns seen in Experiments 1 and 2 we conducted another experiment using only natural soils incubated at 37 °C for 4 d. To ensure independence of isolates by avoiding the same clone, we genotyped a large number of isolates (including those used in previous experiments) using methods and 10 microsatellite markers developed by Grubisha and Cotty (2009) (Supplemental Table S12). We randomly selected independent multilocus genotypes from this pool of isolates, without duplicates of any multilocus genotype. Using these criteria, we compared seven non-aflatoxigenic and 20 aflatoxigenic genotypes, each replicated twice as described above.

Effects of aflatoxin on soil microbial communities:

To determine the effect of aflatoxin on soil microbial communities, we used DNA samples from the natural soils used in Experiment 2 (above). We constructed libraries of the V4 region of bacterial 16S ribosomal RNA and the internal transcribed spacer 1(ITS1) region of fungal ribosomal RNA using methods similar to Kozich et al. (Kozich, Westcott et al. 2013) (Supplemental methods 3).

The library was sequenced at Cornell University's Genomics Facility using the Illumina MiSeq v3 sequencing chemistry (2 X 300 bp reads) on an Illumina Miseq instrument. The 16S rRNA gene and ITS1 amplicon reads were assembled with PEAR (minimum overlap 50 bp, assembly probability 0.001, and PHRED score cutoff of 30), sequencing primers and adapters removed using cutadapt v1.14, and demultiplexed into individual samples with deML (Zhang, Kobert et al. 2014, Renaud, Stenzel et al.

2015) . Operational taxonomic units (OTUs) were assigned using a 3% dissimilarity cutoff and identified using VSEARCH v2.5.2 and PIPITS v1.5.0 pipelines for 16S gene and ITS1 amplicons, respectively (Gweon, Oliver et al. 2015, Rognes, Flouri et al. 2016). Taxonomic affiliations of 16S and ITS1 sequences were performed with the SINTAX algorithm within USEARCH v9.2.64 (sintax cutoff 0.8) using the GreenGenes v13.8 or UNITE v7.2 sequence databases, respectively (DeSantis, Hugenholtz et al. 2006, Abarenkov, Henrik Nilsson et al. 2010, Edgar 2010, Edgar 2016)

Statistics:

Differences in fitness were explained in a series of mixed linear models using main and interaction effects of chemotype, the random effect of isolate nested in chemotype and where applicable: soil sterility, addition of aflatoxin, and temperature. All models to explain differences in fitness were analyzed using ANOVA (Type III) with Satterthwaite approximation. As results from Experiment 1 were different from what we originally hypothesized, we did not expect to see significant differences in Experiment 2 and initially only processed DNA samples from natural soils. Because of this, differences in aflatoxigenic and non-aflatoxigenic isolates from this experiment in natural soil were modeled separately from those in sterile soil. When data from all natural soils incubated at 37 °C without the addition of aflatoxin were combined, the random effect of clone-corrected genotype replaced the random effect of isolate and a categorical grouping variable was added to indicate the experiment from which the data originated.

Transformations using log10() and sqrt() of response variables was necessary in some analyses (See supplemental Tables S1-S9) to equalize variances and linearize the relationship between response and predictor variables. Results were analyzed using R statistics 3.4.0 (R Core Team 2017) Packages 'ARTool' (M Kay and Wobbrock 2016), 'lmerTest' (Kuznetsova, Brockhoff et al. 2016), 'lsmeans' (Lenth 2016), 'tidyverse' (Wickham 2017), and 'Rmisc' (Hope 2013) installed on 21 April 2017.

Microbial communities were analyzed for differences in alpha diversity (species diversity within samples) using an ANOVA (Type III) of the Shannon diversity index as a function of the individual and interaction effects of aflatoxin production of the isolate and the addition of aflatoxin to the soil. Significant deviations in beta diversity (ratio of species diversity between groups) was tested using a PERMANOVA on a Bray-Curtis dissimilarity matrix using 1000 permutations and identical model parameters as specified for alpha diversity tests. Differential abundance of individual taxa between treatments was assessed using DESeq2 with a significance cut off of alpha= 0.05 with the default Bonferroni correction. This analysis was achieved using R packages: 'phyloseq' (P. J. McMurdie 2013), 'ape' (Strimmer 2004), 'ARTool' (M Kay and Wobbrock 2016), and 'DESeq2' (Anders 2014).

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CHAPTER 3

Population subdivision and the frequency of aflatoxigenic isolates in *Aspergillus flavus* across two latitudinal transects in the United States.

Milton T. Drott, Lauren M. Fessler, Michael G. Milgroom

Abstract:

Aflatoxin is an extremely potent hepatotoxin that causes acute toxicosis, cancer, immune suppression, and stunted growth in children. Contamination occurs when maize, peanuts, cottonseed, and tree nuts are infected by *Aspergillus flavus*, the major producer of this toxin; it is more common in the lower latitudes of the United States. However, it is unclear if this increase in contamination is associated with differences in the frequencies of aflatoxigenic individuals at different latitudes. To determine if the frequency of aflatoxin-producing ability of *A. flavus* increases as latitude decreases, we sampled 281 isolates from field soils in two north-south transects in the US and tested them for aflatoxin production. We also genotyped 161 of these isolates using 10 microsatellite markers to assess population structure and to infer the extent of long-distance migration. While the population density of *A. flavus* increased at lower latitudes, there was no difference in the frequency of aflatoxigenic *A. flavus* isolates in relation to latitude. We found that the US population of *A. flavus* is subdivided into two genetically differentiated subpopulations (designated populations A and B) that are not associated with the chemotype or geographic origin of isolates. Our study provides evidence that non-aflatoxigenic individuals may arise by mutation from

aflatoxigenic individuals, as both were sometimes found in the same multilocus microsatellite genotype in population B. Additionally, using the four-gamete-test we found evidence of recombination at all locus pairs for population A, but only at a subset for population B. We conclude that differences in historic and extant recombination may explain the modern subdivision of the *A. flavus* population in the US. Furthermore, as the population subdivision we observed is unrelated to aflatoxin-producing ability, increased aflatoxin contamination in lower latitudes may partially be explained by differences in population density of *A. flavus*, not population structuring. Future study that accounts for the population subdivision observed here is needed to clarify differences between the two populations.

Introduction:

Mycotoxins contaminate approximately 25% of food supplies worldwide (CAST 2003), causing human death and disease from acute toxicosis, cancer, immune suppression, and stunted growth in children (Wild 2007, Liu and Wu 2010, Wild and Gong 2010). Aflatoxin is the most potent mycotoxin known. An estimated 4.5 billion people are chronically exposed to aflatoxin through contaminated food (CDC 2016). Aflatoxin is produced by several species in the genus *Aspergillus* (section *Flavi*), with *Aspergillus flavus* being the main producer (Klich 2007). Aflatoxin often contaminates maize, peanuts, cottonseed, tree nuts, and other seed crops. Within *A. flavus*, there are two morphologically and genetically differentiated phenotypes. These phenotypes were named S and L for small and large sclerotia, respectively (Cotty 1989). All S strain isolates produce aflatoxin, typically in greater quantities than L strain. This

paper only refers to L strain of *A. flavus* because they are the most common in the United States (US). Populations of *A. flavus* are highly diverse (Bayman and Cotty 1991, Horn and Greene 1995) and, importantly, not all isolates produce aflatoxin. Extensive field sampling of *A. flavus* in the US found that only 71% of L strain isolates are aflatoxigenic (Horn and Dorner 1999). Worldwide, both chemotypes, those that produce aflatoxin and those that do not (aflatoxigenic and non-aflatoxigenic, respectively), are often found in soil in the same field (Horn 2003). There is evidence that competition with insects (Drott, Lazzaro et al. 2017) and soil microbes (Drott, Debenport et al. 2018) may maintain both aflatoxigenic and non-aflatoxigenic individuals by balancing selection. While aflatoxigenic isolates have been extensively studied because of the aflatoxin contamination they cause, non-aflatoxigenic isolates have also received attention for their potential as biocontrol agents. When applied to agricultural fields, non-aflatoxigenic isolates compete with aflatoxigenic individuals resulting in a reduction of aflatoxin contamination (Ehrlich 2014). Despite the importance of both aflatoxigenic and non-aflatoxigenic isolates, it remains unclear whether the distribution of *A. flavus* chemotypes correlates with geographic patterns of aflatoxin contamination of crops.

Aflatoxin contamination of crops in the US is more common in the South (Horn 2007). Several studies have suggested that at lower latitudes, greater contamination is caused by a higher frequency of aflatoxigenic individuals (Manabe and Tsuruta 1978, Wicklow and Cole 1982, Wicklow, Dowd et al. 1994, Cotty 1997, Horn 2007, Tran-Dinh, Kennedy et al. 2009). Consistent with this suggestion, Manabe and Tsuruta (1978) reported a gradient in frequencies with latitude in southeast Asia.

However, further evidence for this pattern is less clear. Wicklow and Cole (1982) reported a trend toward increased frequency of aflatoxigenic isolates at lower latitudes despite finding no significant difference. Cotty (1997) found a significant negative correlation in the southern US between latitude and the frequency of aflatoxigenic isolates of *A. flavus* L strain. However, while their sampling sites (located in Arizona, Arkansas, Alabama, Louisiana and Mississippi) may have differed somewhat in climate, they differed only by approximately 3° of latitude (~34° to 31°). In Vietnam, Tran-Dinh et al. (2009) also found a significantly higher frequency of aflatoxigenic isolates at lower latitudes in Vietnam from peanuts, but not from corn and soil or when isolates from all substrates were pooled. Furthermore, not all the evidence supports the prediction of a negative correlation between the frequency of aflatoxin production and latitude. Horn and Dorner (1999) found the frequency of isolates grouped by amount of aflatoxin produced does not appear to be different in the southern and more northern parts of a transect across the US. Despite the dearth of evidence that frequencies of aflatoxigenic and non-aflatoxigenic isolates correlate with latitude, hypotheses have emerged that greater competition with insects and climatic conditions associated with lower latitudes select for aflatoxigenic individuals (Wicklow, Dowd et al. 1994, Horn 2007).

Regional differences in the frequency of aflatoxigenic isolates could be maintained by selection, restricted migration, or both. If migration rates were high relative to differences in selective pressures between regions, frequencies of aflatoxigenic individuals may not differ. Previous studies of the population genetics of *A. flavus* only partially addressed this question. There is some evidence of long-

distance migration of *A. flavus* clones, based on finding isolates of the same vegetative compatibility groups (VCGs) over widespread geographic locations, for example, different provinces of Kenya (Probst, Bandyopadhyay et al. 2011), or different states in the US (Horn and Dorner 1999, Ehrlich, Montalbano et al. 2007, Grubisha and Cotty 2010). However, these examples are not definitive as they either focus on a small subset of VCGs, or do not report chemotypes. It is thus not possible to determine if previous observations of the distribution of VCGs represent infrequent or episodic migration or if migration occurs on a more widespread basis. Making this distinction is necessary for testing the adaptive hypothesis, namely that latitudinal differences in the frequency of aflatoxigenic isolates of *A. flavus* may be maintained by selection.

Comprehensive assessments of migration and population structure in *A. flavus* have been limited, in part, by the use of VCGs, which are thought to represent clones or clonal lineages in *A. flavus* populations (Bayman and Cotty 1993, McAlpin, Wicklow et al. 2002, Ehrlich, Montalbano et al. 2007). The usefulness of VCGs as genetic markers, however, is mitigated by several factors: methods for assaying them are laborious, they exclude large numbers of isolates that are not compatible with known tester isolates, and do not provide information on genetic relatedness between VCGs. In the most extensive sampling of *A. flavus* in the US to date, Horn and Dorner (1998) initially deemed identification of VCGs too labor-intensive, and later identified only VCGs of non-aflatoxigenic isolates (Horn and Dorner 1999). In addition to being biased only to non-aflatoxigenic isolates, their sample was also limited to more common VCGs, as they were only able to match 73 of 126 isolates to tester isolates. In

contrast, studies that have used molecular markers have not been designed to estimate migration of *A. flavus*. Instead, they used isolates from culture collections that were not randomly sampled from specific populations (Geiser, Dorner et al. 2000, Chang, Ehrlich et al. 2006), making inferences about migration difficult. Several of the studies looking for migration focused on small geographic areas (Wicklow, McAlpin et al. 1998, McAlpin, Wicklow et al. 2002). Others did not report whether they found the same genotype in different locations (Barros, Chiotta et al. 2007), or how frequently they found it (Bayman and Cotty 1993, Yin, Lou et al. 2009). Tran-Dinh et al. (2009) found that four of 48 multilocus genotypes sampled from corn collected from markets across Vietnam were found in multiple provinces. However, they did not report which provinces the same genotype was isolated from and did not indicate if they found the same genotype in any of their isolates from peanuts or soil. While these studies indicate that migration happens on a relatively large geographic scale, it remains unclear how important these events are to population structure.

Studies using molecular markers found that aflatoxigenic and non-aflatoxigenic isolates are often found in the same clades (Tran-Dinh, Pitt et al. 1999, Barros, Chiotta et al. 2007, Tran-Dinh, Kennedy et al. 2009), although not in all cases (Bayman and Cotty 1993, Baird, Trigiano et al. 2006). Tran-Dinh et al. (1999) suggest that findings like these indicate that aflatoxin production was lost from aflatoxigenic clonal lineages multiple times, or that recombination has occurred between aflatoxigenic and non-aflatoxigenic lineages. Several studies have found evidence of sexual reproduction in the *A. flavus* genome, even between aflatoxigenic and non-aflatoxigenic lineages. Such recombination is generally thought to be ancient (Moore,

Singh et al. 2009, Olarte, Horn et al. 2012, Moore, Elliott et al. 2013). However, some aspects of modern population structure are consistent with extant recombination. Repeated sampling in the same locations or regions has uncovered novel VCGs each time; this is consistent with recombination between VCGs (Bayman and Cotty 1991, Horn and Greene 1995). There is also evidence that *A. flavus* can reproduce sexually in the field, as a survey of sclerotia produced on inoculated maize ears showed that fertile sexual structures can be found, albeit at a low frequency (Horn, Sorensen et al. 2014). One attempt to look for extant recombination focused on genotyping isolates in a small number of VCGs using microsatellite markers (Grubisha and Cotty 2010). However, mating between VCGs would likely result in recombinants in non-parental VCGs, which were not genotyped, whereas mating within VCGs might be difficult, or impossible, to detect because of limited polymorphisms within any given VCG. Thus, studies looking only within VCGs are unlikely to detect recombination.

The overall objective of this study was to determine if higher levels of aflatoxin contamination in the southern US than in the northern US could be explained in part by differences in the frequency of aflatoxigenic isolates. To address whether structuring occurs based on latitude, we sampled *A. flavus* isolates in two north-south transects in the US, determined their chemotypes and genotyped them with microsatellite markers. Specifically, we addressed the following questions for *A. flavus* populations in the US: 1) Is the frequency of aflatoxigenic isolates negatively correlated with latitude? 2) Is the US population of *A. flavus* genetically subdivided? 3) Is there evidence that both aflatoxigenic and non-aflatoxigenic MLGs migrate

across large geographic distances? 4) Have non-aflatoxigenic isolates arisen multiple times from aflatoxigenic lineages?

Methods:

Isolation and culturing of *Aspergillus flavus*:

Soil was collected between 2013 and 2017 from corn fields in an eastern US transect: Pennsylvania (PA), North Carolina (NC), Florida (FL); and a central US transect: Indiana (IN), Iowa (IA), Oklahoma (OK), and Texas (TX) (Fig. 1; Table S1). Because of the low density of *A. flavus* and difficulty in obtaining isolates in the north, samples from Iowa and Indiana were pooled for all analyses except for assessments of migration (see below). In all states, three fields were sampled between 2016 and 2017, except for North Carolina where two fields were sampled in 2013 and a third was added in 2017. No two fields were within 1 km of each other. We used a sampling scheme that minimized the recovery of multiple *A. flavus* isolates from the same fungal ramet. In each field, we collected 25 independent soil samples, each at least 10 m from any other sample. Each sample contained approximately 50 g from the top 2 cm of soil.

A. flavus was isolated from soil samples on modified dichloran-rose bengal medium using recipes and dilution-plating methods similar to Horn and Dorner (1998). Briefly, 2 g of soil were suspended in 10 ml of 0.2% water agar and 100 ul of this suspension, containing 0.016 g of soil, was used for each dilution plate. Resulting plates were incubated at 30 °C for 3 days. Up to three *A. flavus* colonies were isolated from each soil sample. Conidia from *A. flavus* colonies were streaked onto 25% Czapek Agar (CZA; 2% agar and 12.25 g Czapek medium; Difco Laboratories,

Detroit, MI) and incubated for 16 h at 30 °C in the dark. For each colony, a single hyphal tip was isolated onto full strength CZA to ensure the presence of only one genotype. Isolates were identified as *A. flavus* based on morphology (Horn and Dorner 1998). Only *A. flavus* L-strain isolates, as determined morphologically, were used in this study. Identification was further confirmed for a subset of 90 isolates against closely-related species with similar morphology (e.g., *Aspergillus nomius*) by BLASTn (NCBI) analysis of β-tubulin sequences using similar methods as described previously (Tam, Chen et al. 2014).

Isolates on CZA plates were inoculated into potato dextrose broth (BD Difco, Franklin Lakes, New Jersey) and grown for 4 d at 30 °C. Resulting hyphal mats were harvested by filtration using cheese cloth, rinsed with sterile diH_2O, frozen, lyophilized and stored at -80 °C until DNA was extracted (see below).

Colony-forming units (CFU) of *A. flavus* were counted for four randomly selected soil samples from every field to estimate population density. Differences in population density between geographic regions were assessed using ANOVA and a linear contrast to test for latitudinal differences.

Determination of aflatoxin production:

All isolates were cultured in yeast extract sucrose medium (Horn and Dorner 1999) and on Drosophila culture medium (DCM) to determine aflatoxin chemotype by HPLC, as described previously (Drott, Lazzaro et al. 2017). Cultures on DCM were mechanically damaged with a sterile toothpick to stimulate greater production of aflatoxin (Drott, Lazzaro et al. 2017). Both assays were replicated twice for all isolates. Although *A. flavus* has the potential to produce a variety of mycotoxins, for

example, cyclopiazonic acid (CPA), whose biosynthetic cluster is adjacent to the aflatoxin cluster (Horn, Moore et al. 2009), in this paper we refer to isolates and their chemotype as aflatoxigenic and non-aflatoxigenic based solely on their ability to produce aflatoxin B_1.

The frequency of aflatoxigenic isolates in each field was determined from a sample of isolates all of which originated from independent soil samples. We looked for latitudinal patterns in the frequency of aflatoxigenic isolates using a Chi square test for trends.

DNA extraction and microsatellite genotyping:

DNA was extracted from 4-mm-diameter balls of lyophilized mycelium. Tissue was homogenized in a microcentrifuge tube containing 0.5 and 2 mm zirconia-silica beads and 1 ml of extraction buffer (similar to (Kepler, Humber et al. 2014)) for 6 s on the 4.5 setting of a Thermo Savant Bio101 Fast Prep 120 (Qbiogene, Carlsbad, CA). Extracts were incubated in a boiling water bath for 10 min and centrifuged for 10 min at 10,000 rcf. The supernatant (100 µl) was removed and diluted 10-fold in UltraPure water for use as template DNA for PCR (Thermo Fisher, Waltham, MA).

We selected 10 microsatellite markers described by Grubisha and Cotty (2009) that discriminated among VCGs but had relatively small numbers of alleles within VCGs to avoid hypervariable loci. In addition, to maximize the independence of markers, we chose markers located on different genomic scaffolds of the *A. flavus* genome (Table S2) when possible. To minimize costs, forward PCR primers were modified by adding a 21-bp M13 tail, allowing for the attachment of fluorophores using methods previously described (Schuelke 2000). Fragments were amplified in 20-

μl reactions using methods similar to those of Rafiei et al. (2018). Reactions contained 1 U Native Taq (Thermo Fisher), 400 nM dNTP mix, 2.5 mM MgCl2, 10 μl 2x reaction buffer, 1 μl of template DNA (see above), 250 nM reverse primer, 50 nM forward primer with M13 tail, and 200 nM M13 primer labelled with a fluorophore (either NED, VIC or FAM). Amplification conditions were: 95 °C for 2 min, 35 cycles of 95 °C for 10 s, 58 °C for 20 s, 72 °C for 40 s, with a final incubation at 72 °C for 5 min. Up to three PCR products labeled with different fluorophores originating from the same isolate were pooled by combining 3 μl of each. Pooled samples were mixed with 0.2 μl LIZ500 size-standard (Applied Biosystems, Forester City, CA, USA), and 10 μl highly deionized formamide (Hi-Di formamide; Applied Biosystems). This mixture was denatured at 95 °C for 5 min. Fragment sizes were measured using capillary electrophoresis on an ABI 3730xl DNA Analyzer (Applied Biosystems) at Cornell University Life Sciences Core Laboratories. Resulting fragment sizes were determined using Peak Scanner software (Applied Biosystems).

In order to increase sample sizes of northern sampling locations where we obtained fewer isolates, sometimes multiple isolates originating from the same soil sample were genotyped. In cases where two isolates yielded an identical MLG, only one of them was retained to avoid sampling the same ramet twice.

Analysis of population structure:

Allelic and genotypic diversity, measured as the Shannon diversity index and the Simpson index, corrected for sample size, were determined using ‘poppr’. The genetic relatedness of MLGs was determined from 10 microsatellite markers using calculations of Bruvo’s distance from ‘poppr’ (Kamvar, Tabima et al. 2014). Results

were visualized in minimum-spanning networks (MSNs) using poppr. Data were grouped into *a priori* populations based on state, latitude, or chemotype and tested for population subdivision using analysis of molecular variance (AMOVA) executed in GENALEX v.6.5 (Peakall and Smouse 2006). Population structure was also analyzed *a posteriori* using STRUCTURE v.2.3.4 (Pritchard, Stephens et al. 2000) with a model allowing admixture, a burn-in period of 20,000, and a Markov chain Monte Carlo (MCMC) with 50,000 iterations. This simulation was replicated three times with a range of values for *k*, the number of inferred populations, from 1 to 21, which is the total number of fields sampled. We used structure harvester (Dent and vonHoldt 2012) to infer the number of populations from assessments of Δk described by Evanno (2005). Because STRUCTURE assumes random mating and *A. flavus* populations are known to be clonal (Bayman and Cotty 1991), we also looked for population structuring using the non-model-based multivariate analysis Discriminant Analysis of Principal Components (DAPC) (Jombart 2008) according to procedures outlined in the 'adegenet' tutorial (Jombart 2015). Genetic isolation by distance between states was explored using a Mantel test with 1000 permutations in 'ade4' (Dray and Dufour 2007).

Evidence of recombination:

To look for evidence of recombination, we estimated linkage disequilibrium (LD) in *A. flavus* populations. We calculated the index of association (I_A) and $\bar{r}_d$ in 'poppr' to determine if *A. flavus* populations significantly deviated from random mating. I_A and $\bar{r}_d$ are used as measures of multilocus LD among all loci simultaneously, avoiding issues of multiple comparisons often associated with

estimates of LD between pairs of loci. Significance was determined by comparison to estimates of I_A from 1000 random permutations that simulated random mating.

We calculated the probability that isolates with the same MLG arose independently by recombination (p_{sex}) with poppr using the "multiple" comparison method. This method utilizes binomial equations from Arnaud-Haond et al. (2007) to calculate the probability of finding one more individual with the same MLG as an MLG already in the sample.

We also looked for evidence of recombination using the four-gamete test (Hudson and Kaplan 1985), which is also interpreted as phylogenetic incompatibility in clonal populations (Anderson and Kohn 1998). Pairwise comparisons of all 10 loci used to determine MLGs were assessed for phylogenetic incompatibility using the R package 'FourGamete' developed in this study.

General Statistical Methods:

Results were analyzed using R statistics 3.4.0 (R Core Team 2017) Packages 'poppr' v2.5 (Kamvar, Tabima et al. 2014), 'lme4' (Bates, Mächler et al. 2015) , 'car' (Fox and Weisberg 2011), 'lmerTest' (Kuznetsova, Brockhoff et al. 2016), and 'lsmeans' (Lenth 2016) installed on 21 April 2017. Graphing of results was also completed using R statistics with the packages 'tidyverse' (Wickham 2017), and 'Rmisc' (Hope 2013) installed on the same day.

Results:

Population density and frequency of aflatoxigenic isolates in relation to latitude

The population density of *A. flavus* in soil (expressed as CFU counts) increased significantly from north to south (P<0.001). In both the eastern and central transects

the highest densities were in the most southerly populations (FL and TX), whereas densities were very low in the most northerly populations (PA, IN and IA), and intermediate in the middle (NC and OK) (Fig. 2). Population density increased 100-fold on average from north to south. There was, however, large variation in density even within states, as can be seen by the relatively large standard errors in Fig. 2. In Florida, for example, fields had averages of 5000, 3031, and 78 CFU per g soil. Similar variation was also seen in TX and NC.

Figure 1: Sampling locations of two north-south transects in the United States. The eastern transect fields were in PA, NC, and FL. The central transect fields were in IA & IN, OK, and TX. Three fields were sampled in each state; when only one or two stars are present in a state, then more than one field fell within the area covered by the star. No two fields were within 1 km of each other. Map modified from public domain image: https://commons.wikimedia.org/wiki/File:Blank_US_map_borders.svg.

We obtained 281 isolates of *A. flavus*, each from an independent soil sample, across both transects (Fig. 1, Table S1). Sequences of β-tubulin from 90 isolates identified morphologically as *A. flavus* L strain confirmed their identities. The frequency of aflatoxigenic isolates did not increase significantly with decreasing

latitude in either transect (P>0.61). Indeed, contrary to predictions, the frequency of aflatoxigenic isolates increased with latitude (P=0.023) in the central transect (Fig. 3). While variation in the frequency of aflatoxigenic isolates was high at the state level, when states were pooled within transects, the eastern and central transects were similar, with frequencies of 34% and 39% aflatoxigenic isolates (Fig. 3).

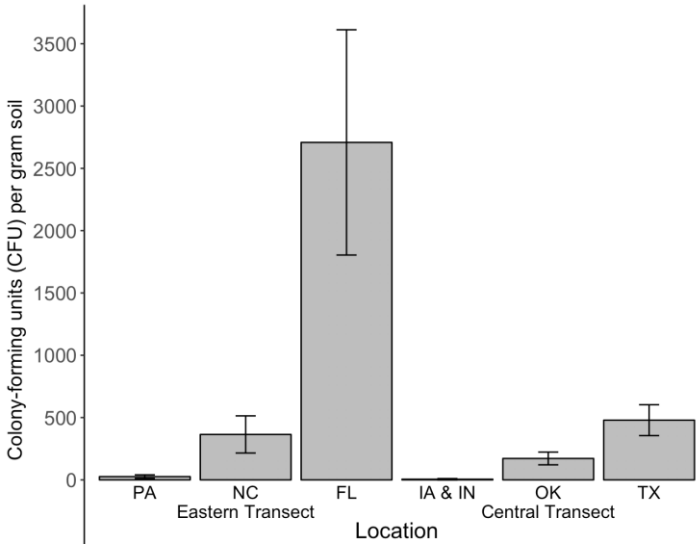

Figure 2: Average population density of *Aspergillus flavus*, expressed as colony forming units (CFUs) per g soil, in two north-south transects in the United States. CFUs were averaged across four soil samples for each of three fields per state. Error bars are ± 1 SE for variation among fields within states.

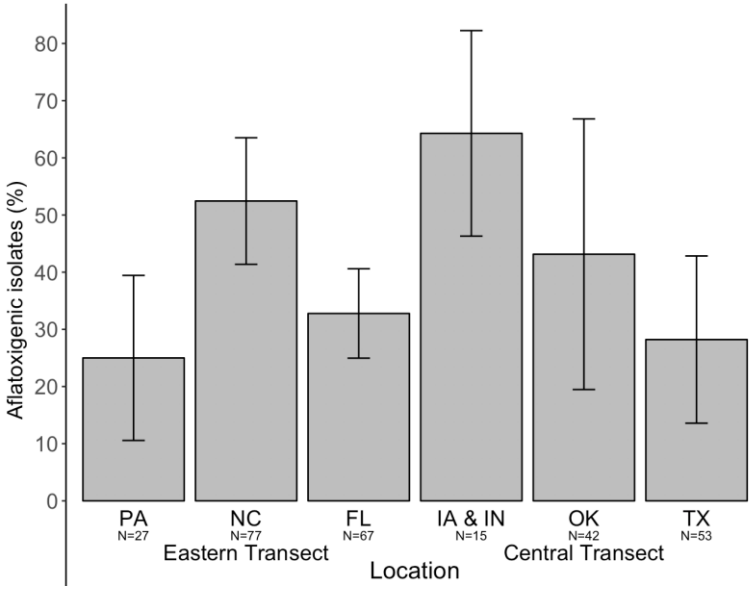

Figure 3: The frequency of aflatoxigenic isolates of *Aspergillus flavus* in two north-south transects in the United States. The frequency of aflatoxigenic isolates was determined from samples of isolates from independent soil samples within each state, with IA and IN pooled (total N=281). Error bars are ± 1 SE for variation among fields within states.

Population subdivision and migration:

We obtained genotypic data on 168 isolates, seven of which were discarded because they originated from a soil sample where an identical MLG had already been sampled. The remaining 161 independent isolates represented 102 unique MLGs, eight of which originated from the same soil sample as another isolate with a different MLG. While closely-related isolates were often from different states and/or different chemotypes (Fig. 4), the US population of *A. flavus* was subdivided into two subpopulations. Analysis of underlying population structure without *a priori* assumptions from STRUCTURE using Δk suggested k=2. Similarly, DAPC inferred two populations (k=2). One population was characterized by distantly related MLGs (population A) while MLGs in the other were more closely-related MLGs (population B); note the thickness of the lines separating MLGs in Fig. 4, indicating genetic distances. Genotypic and allelic diversity were higher in population A than in population B (Table 1). Similarly the clonal fraction of population A (0.041) was markedly lower than that of population B (0.509) (Table 1).

The Mantel test indicated significant isolation by distance of MLGs between states for population A (r=0.186, P<0.001) and population B (r=0.094, P<0.001) (Table 1). In contrast, AMOVA results indicated that nearly all the genetic diversity could be explained within population in comparisons by state for population A (Φ_{PT}=0, P=0.374) and population B (Φ_{PT}=0.005, P=0.285) (Table 1). Consistent with

the lack of genetic differentiation among states, we found nine MLGs of population B in two or more states. However, in population A we only found isolates that shared an MLG twice and both were from the same state (Fig. 4A). In population B, seven MLGs were found in two states (six in NC and FL, and one in FL and TX); one MLG was found in three states (FL, OK, and TX) and another MLG was found in four states IN, IA, TX, OK) (Fig. 4A). These results indicate that clonal migration has occurred between states in population B, but we found no evidence of this in population A, even though the lack of subdivision between states based on AMOVA indicates no restriction on migration. We did, however, observe indications of restricted migration in the form of private alleles, defined here as those occurring only in one state. We observed private alleles at eight of the 10 loci in population A (Table S3) and six of 10 loci for population B (Table S4), with at least one private allele in all but one state for both populations (Table S3 & S4). These results suggest some degree of restricted migration in both populations.

The Mantel test indicated that genetic distance within chemotypes was smaller than between chemotypes for population A (r=0.063, P=0.007), but not for population B (r=0.031, P=0.244) (Table 1). Consistent with this finding AMOVA comparison of chemotypes indicated a small but significant partitioning of genetic diversity between chemotypes of population A (Φ_{PT}=0.059, P<0.001), but not of population B (Φ_{PT}=0, P=0.721) (Table 1).

While there was a small amount of partitioning of genetic diversity by chemotype in population A, aflatoxigenic isolates were often as closely related to non-aflatoxigenic isolates as to other aflatoxigenic isolates in both populations (Fig. 4B).

These results indicate that individuals of both chemotypes in population A are genetically more closely related to other individuals in the same chemotype than they are to those in the other chemotype.

Chemotype was consistent within MLG, with the exception of finding both aflatoxigenic and non-aflatoxigenic isolates in two MLGs in population B (Fig. 4B). One of these MLGs from NC had a single non-aflatoxigenic isolate and four aflatoxigenic isolates. We also observed an MLG containing eight aflatoxigenic isolates and 9 non-aflatoxigenic isolates. In this latter MLG, all toxigenic isolates and a single non-aflatoxigenic isolate were from IA & IN. Non-aflatoxigenic isolates in this MLG were also observed in TX and OK. We did not observe different chemotypes within either of the two instances where multiple isolates were found in the same MLGs, in population A. Finding both chemotypes in a single MLG may be evidence of non-aflatoxigenic isolates arising by mutation within a predominantly aflatoxigenic lineage.

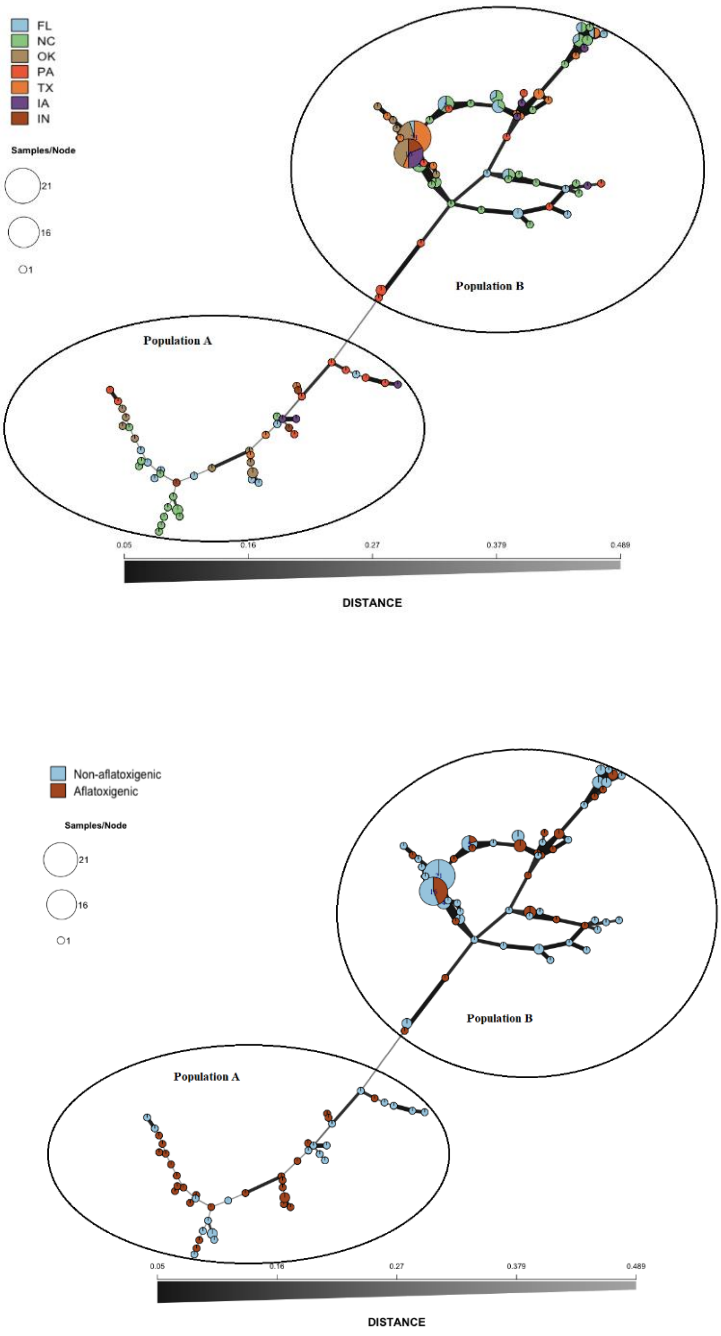

Figure 4: Minimum-spanning network (MSN) representing the genetic relatedness of 161 *Aspergillus flavus* isolates categorized by state of origin (A) and chemotype (B). Assignment of isolates to populations A and B were made by discriminant analysis of principle components (DAPC). Note the thin line separating populations A and B, indicating a large genetic distance. Genetic distances were calculated from 10 microsatellite markers using Bruvo's distance.

Recombination:

The probability that we would sample an identical MLG that arose independently due to random mating and recombination (p_{sex}) ranged from <0.001 to 0.361 in population B. Both estimates of p_{sex} were <0.001 for population A. Because some of the estimates of p_{sex} for population B are high, we must interpret finding the same MLG in different states as sometimes indicating evidence of long-distance migration but cannot rule out the possibility that some MLGs arose recombination.

Estimates of I_A across ten loci were significantly different from expectations of random mating for both populations A and B, although $\bar{r}_d$ was small in both populations indicating weak LD ((I_A= 0.454, $\bar{r}_d$ = 0.0526, P<0.001; I_A= 0.235, $\bar{r}_d$ = 0.0289, P=0.004, respectively; Table 1). Low levels of LD are consistent with a clonal population structure, but do not rule out recombination. Similarly, we found evidence of recombination by observing phylogenetic incompatibility (in the four-gamete test) at all 45 pairwise comparisons between loci for population A, but only at 22 of the 45 pairwise comparisons for population B (Table 1).

Table 1: Comparison of statistics for diversity, population structure, and recombination between population A and population B.

	Population A	Population B
N	49	112
Allelic diversity	0.863	0.434
Genotypic diversity	0.998	0.941
Clonal fraction	0.041	0.509
Population subdivision: chemotype	Φ_{PT}=0.059, *P*<0.001	Φ_{PT}=0, *P*=0.721
Population subdivision: state	Φ_{PT}=0, *P*=0.374	Φ_{PT}=0.005, *P*=0.285
Isolation by distance	*r*=0.186, *P*=0.001	*r*=0.094, *P*=0.001
Isolation by chemotype	r=0.063, P=0.007	r=0.031, P=0.244
Total alleles at all loci	134	38
Loci with private alleles	8	6
Total private alleles at all loci	33	11
Linkage disequilibrium	I_A= 0.454, r_d = 0.0526, *P<0.001*	I_A= 0.235, r_d = 0.0289, *P=0.004*
Phylogenetic incompatibility (four-gamete test)	45/45	22/45

Discussion

We found evidence that *A. flavus* in the US is subdivided into two populations that cannot easily be explained by geography or chemotype. While population subdivision has previously been observed within *A. flavus* (Geiser et al. 1998), this subdivision was later determined to reflect differences between S and L strains (Geiser et al. 2000). Subsequent studies also showed such subdivision within the L strain (Geiser, Dorner et al. 2000, Chang, Ehrlich et al. 2006); our findings emphasize the extent and distribution of subdivision in natural populations. Although there has been speculation about the selective pressures that maintain higher frequencies of aflatoxigenic isolates in warmer, southern latitudes of the US (Wicklow, Dowd et al. 1994, Horn 2007), we did not observe this pattern. While the population density of *A. flavus* was significantly greater in the south, the frequency of aflatoxigenic isolates

was not different from north to south. Within a population, isolates were often as closely related to other isolates from different states but we did find evidence of isolation by distance. Genetic distance within chemotypes was smaller than between chemotypes for population A but not population B. In both populations isolates of different chemotypes were closely related. Migration of both aflatoxigenic and non-aflatoxigenic MLGs was common between states in population B. We observed the same MLGs across multiple locations only in population B, which may serve as evidence of long-distance migration, although some of these MLGs may arise by chance through recombination. However, we speculate that recombination in this population is unlikely, as evidence of recombination in the form of phylogenetic incompatibility was only observed between 22 of 45 possible locus pairs. In population A, however, phylogenetic compatibility was observed at all pairwise locus comparisons. We speculate that recombination may be more common in population A, explaining higher diversity of MLGs and making it more difficult to observe the same MLG twice. In addition to signs of recombination, we observed non-aflatoxigenic and aflatoxigenic isolates in the same MLG only in population B. This may indicate that non-aflatoxigenic lineages arise by mutation from aflatoxigenic lineages. We believe that the most parsimonious explanation for these observations is the presence of low levels of extant recombination within and between clonal lineages of population A, while population B may not be recombining as much. Our results suggest that greater aflatoxin contamination found in the southern US is more likely explained by higher population densities of *A. flavus* than by differences in the frequency of aflatoxigenic individuals.

Previous studies indicated that there were higher levels of aflatoxin contamination in warmer, lower latitudes of the US (Cotty and Jaime-Garcia 2007), leading to speculation that aflatoxigenic fungi are adapted to warmer climates and increased abundance of insects (Wicklow, Dowd et al. 1994, Horn 2007). While our eastern and central transects spanned 11.5° (42.1° to 30.6°) and 9.6° (40.2° to 30.6°) of latitude, respectively, we did not observe any evidence of a correlation between frequency of aflatoxigenic *A. flavus* and latitude (Fig. 3). Therefore, our results do not support hypotheses that selection maintains a geographic pattern (Wicklow and Cole 1982, Wicklow, Dowd et al. 1994, Cotty 1997, Horn 2007). Rather, our findings are consistent with those of Horn and Dorner (1999) who found no clear latitude-related pattern in the frequency of isolates producing different amounts of aflatoxin. Their transect, however, was characterized by sampling mostly east to west (~28° longitude) in the southern US and captured less variation in latitude (approximately 6.5°, from ~31.5 to ~38) than our study. In contrast, our study examined two replicated north-south transects including a range of areas, from those with relatively little aflatoxin contamination to those with frequent high levels of contamination. Ours is a relatively comprehensive assessment across latitudes, and yet we found no differences in the frequency of aflatoxigenic isolates.

In contrast to the frequency of aflatoxigenic isolates, the overall density of *A. flavus* in soil was markedly greater at lower latitudes (Fig, 2). Indeed, our results suggest a northern limit to the range of *A. flavus* in the US, although there are some reports of the fungus at higher latitudes than those sampled here (Klich 2002). Despite repeated sampling, we were unable to isolate *A. flavus* from soil from several fields in

Ithaca and Aurora, New York (both at ~42.5° latitude) (results not shown). The geographic patterns we found for population density are consistent with a suppressive soil effect observed by Drott et al. (2018) under laboratory conditions: namely, soils incubated at cooler temperatures are more inhibitory to the growth of *A. flavus* than those incubated at warmer temperatures. It has been shown that stress on crops associated with both drought (Jones, Duncan et al. 1981) and high temperature (Abbas, Williams et al. 2002) results in greater aflatoxin contamination. Consistent with these observations, Cotty and Jaime-Garcia (2007) suggest that crops grown in warmer climates will more frequently be infected by *A. flavus* and have higher amounts of aflatoxin contamination. We speculate that greater aflatoxin contamination at lower latitudes (Horn 2007) is likely caused by an interaction between the higher population density of *A. flavus* and the effect of climate in warmer, lower latitudes on the susceptibility of crops to aflatoxin contamination.

As we did not see any geographic pattern in the frequency of aflatoxigenic isolates (Fig. 3), we also observed no population structure based on microsatellite genotyping that could easily be explained by geography or chemotype. Migration between areas could account for mixing of MLGs and chemotypes. We found that nine MLGs, accounting for more than half of the isolates in population B, were sampled from more than one state (Fig. 4A). By contrast, in population A we did not observe any MLG in more than one state, perhaps indicating that this population is less likely to migrate than population B. It is also possible, however, that given the higher allelic diversity in population A, this difference reflects the probability of sampling the same MLG twice instead of indicating differences in migration. The

difference in diversity between the two populations is illustrated by our finding that while both populations are diverse (Fig 4.), with 47 and 55 MLGs (populations A and B, respectively), but population B has a much higher clonal fraction (0.51) than population A (0.041). In addition to significant isolation by distance, both populations A and B had private alleles, together suggesting that there is some level of restricted migration occurring in each population. Consistent restricted migration, both populations had significant isolation by distance. However, our AMOVA results suggest that there was no genetic differentiation based on location, consistent with frequent migration. Migration over large geographic areas is commonly observed in some plant pathogenic fungi (Brown and Hovmøller 2002). However, the most comprehensive assessment of migration in *A. flavus* prior to this study was based on VCGs of a small subset of non-aflatoxigenic isolates and did not address underlying population structure (Horn and Dorner 1999). Our study suggests that migration of *A. flavus* is common, despite some restriction.

While migration may mix different aflatoxigenic and non-aflatoxigenic genotypes across large geographic distances, it has remained unclear whether recombination could account for some of the diversity observed within *A. flavus* chemotypes. As discussed above, many aspects of *A. flavus* population structure are known to be consistent with recombination. Ancient recombination, even between aflatoxigenic and non-aflatoxigenic lineages, shaped the *A. flavus* genome (Moore, Singh et al. 2009, Olarte, Horn et al. 2012, Moore, Elliott et al. 2013). We found phylogenetic incompatibility, a sign of recombination, in all 45 pairwise comparisons of 10 loci for population A, but only in 25 of the comparisons for population B. As we

chose microsatellite markers with the fewest reported alleles and least polymorphism within VCGs to avoid hypervariability, this finding is more consistent with an interpretation of phylogenetic incompatibility as representing recombination rather than homoplasy of hypervariable loci. Differences in the number of loci at which we find incompatibility may suggest that recombination is more common in population A than in population B. However, low diversity in population B might make it difficult to observe recombination as recombination between individuals with identical alleles cannot be detected in our analysis. Consistent with sex being more common in population A, this population had markedly smaller clonal fraction than population B (0.04, 0.509 respectively). Additionally, the allelic diversity of population A was much higher than that of population B despite larger sample size in the latter. We speculate that such a pattern could reflect extant recombination in population A and only historic recombination in population B.

Consistent with the characteristically clonal nature of *A. flavus* populations (Bayman and Cotty 1993, McAlpin, Wicklow et al. 2002, Ehrlich, Montalbano et al. 2007), we found the same MLG in different soil samples, and our assessment of linkage disequilibrium (LD) indicates that neither of the US populations of *A. flavus* is mating randomly. However, this does not rule out the possibility that sex occurs infrequently in either population. Extant recombination, however, has not been extensively studied. Notably, Grubisha and Cotty (2010) did not find evidence of recombination within or between three geographically widespread VCGs. However, as we emphasized above, genotyping only isolates in the most common VCGs limits the ability to detect recombination. We speculate that many of the VCGs that have

compatible tester isolates are likely from population B where the probability of sampling the same MLG is much higher. As recombination may be less common in population B, studies focused on this population may be less likely to find recombination. Such recombination may allow for the origin of new lineages of both chemotypes, potentially explaining some of the diversity we observed.

In addition to the possibility of recombination, the clonal aspect of *A. flavus* ecology may also help to explain how non-aflatoxigenic lineages arise. It is known that non-aflatoxigenic lineages descended from aflatoxigenic genotypes in the evolutionary history of *A. flavus*, as indicated by the presence of partial aflatoxin gene clusters in many non-aflatoxigenic lineages (Chang, Horn et al. 2005). Examination of the MSN coded for chemotype (Fig. 4B) shows a mosaic of aflatoxigenic and non-aflatoxigenic isolates throughout the network in both populations A and B. Although, in population A. isolates within chemotype were more closely related to each other than to isolates of a different chemotype. This result is consistent with other studies that found that isolates of different chemotypes are often found in the same clade of phylogenetic analyses (Tran-Dinh, Pitt et al. 1999, Barros, Chiotta et al. 2007, Tran-Dinh, Kennedy et al. 2009). Moreover, we found both aflatoxigenic and non-aflatoxigenic isolates in population B within the same MLG. We speculate that we did not make a similar observation for population A because of the higher diversity of MLGs, perhaps from recombination, in this population. These findings serve to emphasize that aflatoxigenic and non-aflatoxigenic isolates may be very closely related and suggest that non-aflatoxigenic isolates may arise relatively frequently in extant populations through mutation.

Our results help to explain patterns of aflatoxin contamination in the US while also providing information on the population structure of *A. flavus* that informs efforts to mitigate contamination. We speculate that greater aflatoxin contamination in the southern US (Horn 2007) is more likely related to higher population densities of *A. flavus* than to higher frequencies of aflatoxigenic isolates. This trend in population density is consistent with increased suppression of *A. flavus* growth in soils incubated at cooler temperatures (Drott, Debenport et al. 2018). The population structuring we observed could not readily be explained by differences in geography or chemotype. We speculate that differences between the two populations may be explained by differences in recombining ability. Namely, Population B may comprise a small number of closely related clonal lineages interacting without sex, while lineages in population A may be more likely to outcross. While it is unclear what may drive the genetic isolation of these two populations, it does not appear to be related to chemotype. Both populations contained aflatoxigenic and non-aflatoxigenic isolates with no discernable pattern across states. Our results suggest that selective pressures acting on aflatoxin production occur in both populations at the field level instead of on a broad geographic scale as had previously been suggested (Manabe and Tsuruta 1978, Wicklow and Cole 1982, Wicklow, Dowd et al. 1994, Cotty 1997, Horn 2007, Tran-Dinh, Kennedy et al. 2009). Indeed, competition between *A. flavus* and insects (Drott, Lazzaro et al. 2017) or soil microbes (Drott, Debenport et al. 2018) may maintain both chemotypes on a local scale. We speculate that even when selective pressures in a local area favor one chemotype, migration observed for some MLGs in this study may maintain both chemotypes locally. Future work that accounts for the observed

population subdivision is needed to better define differences between these two populations, particularly with respect to recombination and migration. Furthermore, they highlight the risk that biocontrol isolates of *A. flavus*, or at least their genes, have the potential to become part of the US population (or gene pool) on larger geographic scales than previously thought.

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