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

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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. 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 0–1750 ppb aflatoxin, using quantitative PCR 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 threefold 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, to our knowledge, provide the first clear evidence of a fitness advantage conferred to *A. flavus* by aflatoxin when interacting with insects.

1. Introduction

Despite recognition in the literature of the vast diversity and prevalence of microbial secondary metabolites [1], 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 [2]. Gould & Lewontin [3] warned that such misappropriations of current use 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 [4], it is now commonly thought that sub-inhibitory concentrations are the norm [5]. At these concentrations, many 'antibiotics' have exhibited hormesis. Antibiotic targets of inhibition are now seen as signalling receptors [5–7]. Although work on antibiotics as signalling molecules has been extensively reviewed [1,2,6,7], 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 in [8–11]). 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 [12]. Nevertheless, as with antibiotics, connecting functionality in the laboratory to adaptive hypotheses about secondary metabolism has proved difficult. Part of this difficulty is in separating the potential benefit of a small molecule from other pleiotropically linked fitness traits [13]. 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 [14]. In addition, the expression of other secondary metabolites is affected by this regulatory gene (reviewed in [15]). 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 [16].

Recently, several studies have attempted to show the ecological role or selective forces driving the evolution of fungal secondary metabolism, often 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*. [17] 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 [18–20]. In addition, some specific fungal compounds may have a direct role in inhibiting fungivory [21,22]. 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*, *Aspergillus 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 [23–26]. However, not all strains of *A. flavus* produce aflatoxin. Extensive field sampling of *A. flavus* in the USA found that 29% of all isolates were non-aflatoxigenic [27]. Worldwide, both chemotypes (aflatoxigenic and non-aflatoxigenic) are often found in the same field [28]. 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 [29]. However, the selective forces that drive the balancing selection for aflatoxin production have remained a mystery.

Janzen [30] elaborated the hypothesis that aflatoxin production is favoured 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 favours non-producers, thereby driving balancing selection. In the decades since, the amount of research on insect–aflatoxin interactions has eclipsed research on other potential competitors. Many studies have demonstrated that pure aflatoxins added to food sources are toxic to a wide range of insects [20,31–33], although the degree of toxicity varies greatly even within a genus [33]. 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*. [34] speculated that the increasing toxicity to the European corn borer of compounds along the aflatoxin biosynthetic pathway [35] 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 favoured 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*. [17]. 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: (i) does aflatoxin decrease the fitness of *Drosophila* when it is added directly to food? (ii) does aflatoxin increase the fitness of *A. flavus* in the presence of *Drosophila* larvae when it is added to a nutrient source? (iii) do aflatoxigenic isolates of *A. flavus* have higher fitness compared to naturally occurring non-aflatoxigenic isolates when interacting with *Drosophila*? and (iv) does physical damage by *Drosophila* larvae to *A. flavus* result in an increase in aflatoxin production?

2. Material and methods

(a) Cultures of *Aspergillus flavus* and *Drosophila*

Field isolates of *A. flavus* used in experiments were obtained previously (electronic supplementary material, 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 days. Spores were harvested in sterile deionized H_2O (diH_2O) with 0.05% Tween 20 and counted on a haemocytometer. Although *A. flavus* has the potential to produce a variety of mycotoxins, e.g. cyclopiazonic acid, 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, per litre diH_2O: 50 g of yeast, 70 g of yellow cornmeal, 40 g of glucose, 7 g of agar, and 1 ml of a solution with 4.2% phosphoric acid and 42% propionic acid to control microbial growth. To harvest *Drosophila* larvae, adult flies were transferred to fresh medium and allowed to lay eggs for 16 h. Resulting eggs were removed using an artist's paintbrush, 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.

(b) Experimental microcosms

All experiments were conducted in 2 ml microcentrifuge tubes containing 200 µl of a modified *Drosophila* culture medium (DCM) [17]. The medium contained 57.2 g of sucrose, 57.2 g of sieved cornmeal (particles <0.25 µm in size) and 57.2 g of brewer's yeast per litre diH_2O. When applicable, aflatoxin B1 (Sigma-Aldrich A6636) was dissolved and diluted into a standardized volume of methanol, which was added to molten DCM after autoclaving. A sterile toothpick was used to macerate solidified medium, increasing the surface area for greater colonization by *A. flavus*. Without maceration, burrowing of larvae greatly increased the surface area of the medium, resulting in greater fungal fitness relative to tubes without larvae. 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 a 70 spores μl^{-1} 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 D : 12 L cycle under a fluorescent lamp on a laboratory 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.

(c) High performance liquid chromatography 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 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 s. Resulting suspensions were centrifuged at 13 800*g* for 8 min, and 450 µl of aflatoxin extract was combined with 1440 µl of diH_2O 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 the 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 high performance liquid chromatography (HPLC) (Santa Clara, CA, USA) in comparison with standard curves constructed from analytical standards (Sigma-Aldrich CRM44647, St Louis, MO, USA) diluted to 25% MeOH in the range of 8–0.0008 ng μl^{-1} (8000–0.8 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 4.6 × 250 mm column (Agilent). Injection of 50 µl was run at 1 ml min^{-1} at 45°C for 15 min. As we only quantified aflatoxin B1, all subsequent references to 'aflatoxin' refer to this form of the toxin.

(d) Quantitative polymerase chain reaction 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 [36]. After adding 1 ml of extraction buffer, samples were homogenized in a Fast Prep 120 set to 6.5 for 45 s. 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 s 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 3 M NaOAc (pH 5.5) was added and mixed by vortexing for 5 s. DNA was precipitated in two volumes of −20°C ethanol by vortexing for 5 s 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 s 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 10 mM Tris-HCl (pH 8.0) 0.1 mM EDTA (TE).

We developed quantitative polymerase chain reaction (qPCR) primers by aligning sequences of the *O*-methyltransferase gene (*omtA-1*) of the aflatoxin biosynthesis cluster sequences from GenBank (accession numbers, electronic supplementary material, 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 from the Aflo8 primers with those from primers we developed for the *A. flavus* actin gene (*Act1*) (see the electronic supplementary material). Results from the two primer sets were highly correlated ($r = 0.966$).

qPCRs 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 of DNA template and 8 µl of ultrapure water. PCR cycling conditions were: 95°C for 5 min, 40 repeats of 95°C for 20 s and 64.2°C for 30 s. Melt-curve analysis was done in 0.5°C increments between 60 and 95°C after 10 s at 95°C.

Aspergillus 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}). All standard curves indicated 93–99% efficiency with r^2 values greater than 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.

(e) 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 to 4000 ppb, in increments of 500 ppb, to the medium. At each concentration, 10 microcosms were observed microscopically every 24 h for 15 days. The number of pupated or emerged flies was recorded.

The number of pupae and the number of emerged flies observed in a given tube after 15 days were analysed against aflatoxin content in the food using a simple linear regression.

Emergence data were $\log_{10}$ transformed because of unequal variance in the arithmetic scale.

(f) 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 and 1750 ppb aflatoxin. Six field isolates of *A. flavus* (three aflatoxigenic and three non-aflatoxigenic) were grown in a fully factorial design, 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.

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 ($n = 84$). To allow for some larvae to begin pupation, facilitating the differentiation of the *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 diH_2O was added and vortexed for 5 s. The tube contents were deposited onto a Petri dish, diluted 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.

Mixed linear models were constructed to explain differences in fungal and larval fitness as a function of main and interaction effects of: the quantitative variables of toxin added to the food, larvae/no-larvae and 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.

(g) 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 exogenous aflatoxin as added in previous experiments. The design was fully factorial, with larvae and no-larvae treatments. DNA and aflatoxins were quantified from three pooled tubes as described above.

A mixed linear model was constructed to explain differences in 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 non-toxigenic isolates were compared within larvae and within no-larvae treatments.

(h) 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 a *Drosophila* larva 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.* [22]. The resulting damage to colonies resembled the 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 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.

(i) General statistical methods

Results were analysed using R statistics v. 3.4.0 [37] packages 'lme4' [38], 'car' [39], 'lmerTest' [40], 'lsmeans' [41], 'tidyverse' [42] and 'Rmisc' [43] installed on 21 April 2017.

3. Results

(a) Experiment 1: effect of aflatoxin on *Drosophila* fitness

Aflatoxin significantly ($p < 0.0001$) decreased the number of *Drosophila* pupae that formed (figure 1*a*) as well as the number of adult flies that emerged (figure 1*b*). 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. This suggests that the insects did not consume the food at higher aflatoxin concentrations. Melanization (electronic supplementary material, 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).

(b) 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 the 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

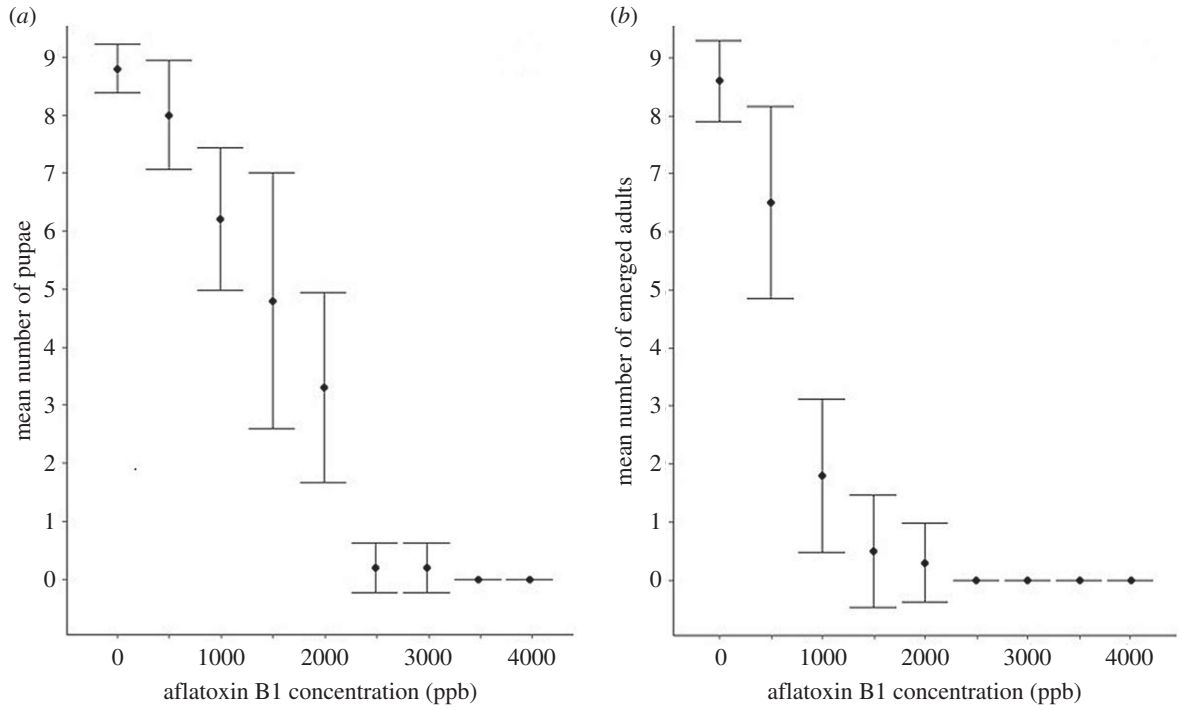

Figure 1. Dose–response curves of *Drosophila* fitness as a function of aflatoxin B1 added to the medium. The number of pupated larvae (*a*) or subsequently emerging adult flies (*b*) was tallied over 15 days. All points are the average of 10 replicated tubes, each containing nine larvae at a given aflatoxin concentration ($n = 90$). Error bars represent $\pm$s.d. Aflatoxin significantly decreased both pupation and emergence of larvae ($p < 0.0001$).

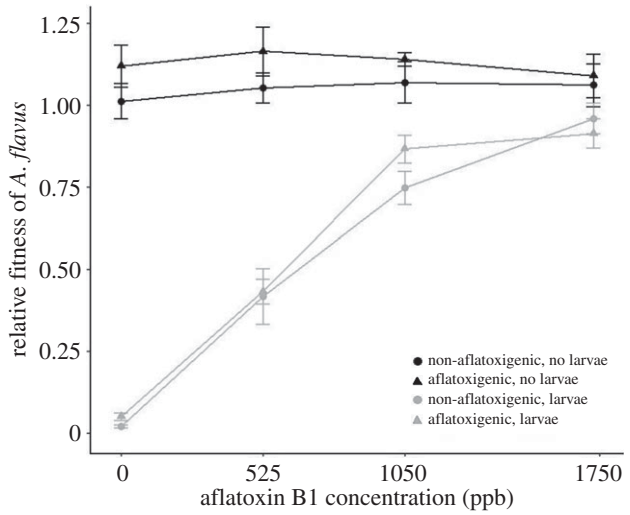

Figure 2. Average relative fitness of three aflatoxigenic and three non-aflatoxigenic isolates of *Aspergillus flavus* with and without *Drosophila* larvae incubated for 72 h. Fitness of *A. flavus* was estimated by qPCR. Each isolate was replicated three times with and without *Drosophila* larvae at each aflatoxin concentration ($n = 144$). Error bars represent $\pm$s.e. 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 ($p = 0.2066$).

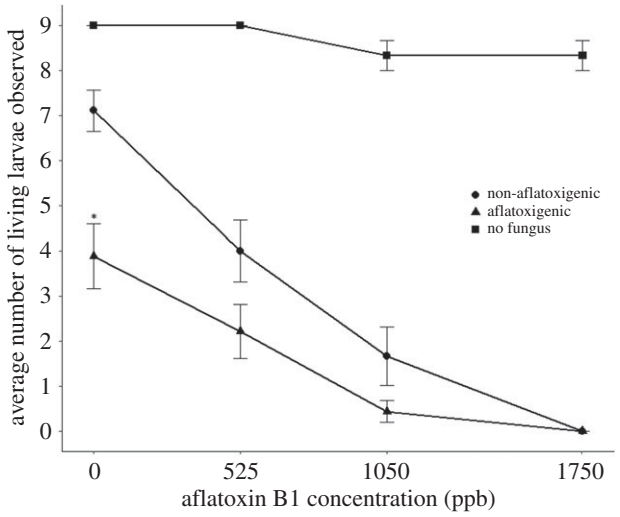

Figure 3. Average number of living larvae observed in microcosms after 96 h with either aflatoxigenic, non-aflatoxigenic fungus or no *Aspergillus flavus*. Means are based on three independent replicates of each of three isolates in each fungal treatment or three replicates of no-fungus treatment for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

linearly relative to controls in toxigenic and non-toxigenic fungal treatments (figure 3).

(c) Experiment 3: effect of aflatoxigenicity in *Aspergillus flavus* in the presence of *Drosophila* larvae

We found no significant interaction determining the 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 non-toxigenic) (figure 4).

(d) 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

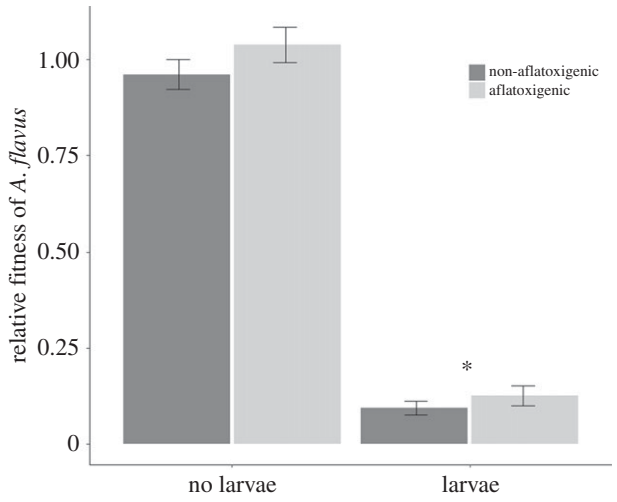

Figure 4. Average fitness of nine toxigenic and nine non-toxigenic isolates of *Aspergillus flavus* with and without competition from *Drosophila* larvae. Fitness was estimated by qPCR. Each isolate was replicated three times in each treatment ($n = 108$). Error bars represent $\pm$s.e. When flies were added, fitness was slightly higher for toxigenic isolates than for non-toxigenic isolates ($p = 0.033$ for the one-sided hypothesis test that toxigenic isolates have greater fitness than non-toxigenic, indicated with an asterisk).

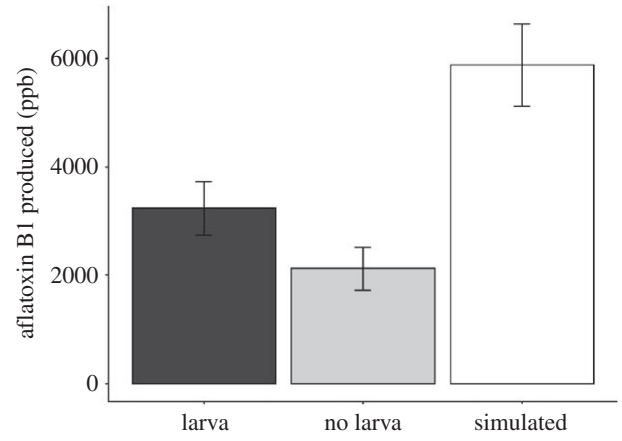

Figure 5. Average aflatoxin production of 12 toxigenic field isolates of *Aspergillus flavus* subjected to damage from a larva, no damage (no-larva) or simulated damage using a toothpick over the course of 72 h. Each isolate was replicated three times within each treatment ($n = 108$). Error bars represent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

5877 ppb aflatoxin, which was significantly higher than the average for the 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 ($p = 0.4556$).

4. 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 *A. flavus* isolates ranged from 8 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 defence 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 with insects. We assume that a cost of aflatoxin production in the absence of susceptible insects will favour 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 confer 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 [20,31–33]. Kroymann *et al*. [44] 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 in the aflatoxin gene cluster [29,45] could be driven by 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 2 and 3, 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 (M. T. Drott 2017, 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*. [34] speculated that geographical distributions seem to favour aflatoxigenic individuals in warmer, lower latitudes [27,46] that may coincide with the increased threat of fungivory from insects.

The biosynthesis of aflatoxin is energetically costly [13,47,48]. Given finite resources, the allocation cost of diverting energetic resources to defence has been demonstrated in several systems [49]. Thus, the allocation of resources to the production of aflatoxin when insects are absent 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 that this contradicts the hypothesis that aflatoxin production is costly to fitness, however, because the cost of secondary metabolites

is often measurable only in competitive, high-stress, low-resource situations [50] the cost of aflatoxin production could be hidden by the nutrient-rich synthetic medium we used for our experiments. A more competitive experimental set-up was not feasible in our study owing 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*. [34] described Aspergilli in general as colonizing substrates in and on the soil, with little growth through the soil itself (similar to *Penicillium* growth pattern; [51]). 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 in [52]), 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 [53]. 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 *Aspergillus nidulans* with a concurrent decrease in insect fitness [21,22]. 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 [21,22]. 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 [54,55]. Finally, cellular damage caused by physical maceration is known to cause the release of reactive oxygen species [56], which have been associated with increased aflatoxin production [57,58]. 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 & Lewontin's [3] 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 an 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 the 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 [30] hypothesis that aflatoxin mediates interference competition. *Aspergillus 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 [59] inhibits fungivory, whereas aflatoxin secreted from the fungus into the substrate [60,61] 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 the electronic supplementary material, video S1), 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 owing to accumulation in fungal cells before secretion.

The 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 [62–64]. 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 could benefit from aflatoxin secreted by toxigenic individuals when co-occurring 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 [65]. 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, to our knowledge, 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 favoured 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 [30] 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.

Data accessibility. All data are available at Dryad: http://dx.doi.org/10.5061/dryad.pq365 [66].

Author contributions. M.T.D. and M.G.M. conceived of the study and designed experiments. M.T.D. performed experiments and statistical analyses. M.T.D., B.P.L. and M.G.M. contributed to the writing of the manuscript. B.P.L., D.L.B. and I.C. contributed organisms, materials and/or analytical tools.

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