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Research Paper

Aflatoxin Contamination of Dried Insects and Fish in Zambia

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ABSTRACT

Dried insects and fish are important sources of income and dietary protein in Zambia. Some aflatoxin-producing fungi are entomopathogenic and also colonize insects and fish after harvest and processing. Aflatoxins are carcinogenic, immune-suppressing mycotoxins that are frequent food contaminants worldwide. Several species within *Aspergillus* section *Flavi* have been implicated as causal agents of aflatoxin contamination of crops in Africa. However, aflatoxin producers associated with dried fish and edible insects in Zambia remain unknown, and aflatoxin concentrations in these foods have been inadequately evaluated. The current study sought to address these data gaps to assess potential human vulnerability through the dried fish and edible insect routes of aflatoxin exposure. Caterpillars ($n = 97$), termites ($n = 4$), and dried fish ($n = 66$) sampled in 2016 and 2017 were assayed for aflatoxin by using lateral flow immunochromatography. Average aflatoxin concentrations exceeded regulatory limits for Zambia (10 μg/kg) in the moth *Gynanisa maja* (11 μg/kg), the moth *Gonimbrasia zambesina* (Walker) (12 μg/kg), and the termite *Macrotermes falciger* (Gerstacker) (24 μg/kg). When samples were subjected to simulated poor storage, aflatoxins increased ($P < 0.001$) to unsafe levels in caterpillars (mean, 4,800 μg/kg) and fish (*Oreochromis*) (mean, 23 μg/kg). The L strain morphotype of *A. flavus* was the most common aflatoxin producer on dried fish (88% of *Aspergillus* section *Flavi*), termites (68%), and caterpillars (61%), with the exception of *Gynanisa maja,* for which *A. parasiticus* was the most common (44%). Dried fish and insects supported growth (mean, 1.3×10^9 CFU/g) and aflatoxin production (mean, 63,620 μg/kg) by previously characterized toxigenic *Aspergillus* section *Flavi* species, although the extent of growth and aflatoxigenicity depended on specific fungus-host combinations. The current study shows the need for proper storage and testing of dried insects and fish before consumption as measures to mitigate human exposure to aflatoxins through consumption in Zambia.

Key words: Aflatoxin; *Aspergillus*; Fish; *Gonimbrasia*; Insects; Zambia

Insects are an important food and income source in Zambia, providing dietary protein and supplementing incomes in rural and urban areas *(25–28, 39)*. Edible insects are highly nutritious, being comparable to or better than common sources of meat such as chicken and beef and yet they are less expensive *(40)*. More than 60 insect species are consumed in Zambia, with the most popular being lepidopteran caterpillars in the order Saturniidae, grasshoppers, and termites *(44)*. The most common species of caterpillars include the mopane worm, *Gonimbrasia belina* (Westwood) (local name "mumpa"), *Gonimbrasia zambesina* (Walker) (local name "mumpa"), and *Gynanisa maja* (Klug) (local name "chipumi") *(10, 28, 40)*. *Macrotermes falciger* (Gerstacker) (local name "inswa") and *Ruspolia differens* (Serville) (local name "nshonkonono") are the frequently consumed termite and grasshopper species, respectively *(40)*. In Zambia, insects are harvested in rural areas and sold in urban centers, making concerns over their safety relevant to the wider population.

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Although the importance of insects in human diets worldwide is well known *(11, 43)* and expected to rise owing to demands from the increased population *(43)*, concerns about the safety of insects as human food have also risen *(31)*. Insects could be contaminated with hazardous microbes and mycotoxins such as aflatoxins *(31, 43)*. Aflatoxins are cancer-causing, immunosuppressive mycotoxins that are associated with stunting, reduced weight gain, and rapid death *(14, 23, 24, 33, 35, 41, 45)*. Enforcement of aflatoxin regulatory limits in foods and feeds results in loss of markets and reduced income *(42, 47)*. Aflatoxins are produced by several species in *Aspergillus* section *Flavi*. The fungi disperse from soil, organic matter, and alternative hosts to crops, trees, animals, and foods. Species most notorious for contaminating foods with aflatoxins are *Aspergillus flavus* (produces only B aflatoxins) and *A. parasiticus* (produces both B and G aflatoxins) *(9, 17, 34)*. However, recent work has revealed that the causal agents of aflatoxin contamination actually include several other species. *A. flavus* is typically divided into L and S morphotypes based on sclerotia size and habit. The L morphotype produces few large (average diameter, >400 μm) sclerotia, and the S morphotype produces numerous

small (average diameter, <400 μm) sclerotia *(5)*. Several species in addition to *A. flavus* have S morphology. Both the S morphotype of *A. flavus* and the other S morphology aspergilli consistently produce large quantities of aflatoxins. The phylogenetically delineated S morphology taxa include (i) *A. flavus* S strain, (ii) lethal aflatoxicosis fungus S_B that severely contaminated maize and led to many deaths in Kenya *(33),* (iii) the unnamed taxon S_{BG} from West Africa *(7),* and (iv) *A. minisclerotigenes (32)*. Aflatoxin-producing fungi have been isolated from insects and fish *(1, 19, 31),* and these fungi can infect, and sometimes kill, live insects *(12, 38)*. Aflatoxigenic fungi may also become associated with dried fish and insects through poor processing, such as sun drying on the ground or in open environments, which is a common practice in Zambia *(27)*. Soils in cultivated and uncultivated areas of Zambia contain aflatoxin-producing fungi known to contaminate crops *(20)*. It is likely that these fungi also have the ability to produce aflatoxins in fish and insects *(1, 19, 31)*. *Aspergillus* species and genotypes vary in average aflatoxin-producing potential, and the relative importance of specific etiologic agents may vary among regions *(9)*. To assess the extent to which mitigation may be required, it is important to characterize aflatoxin concentrations and frequencies of aflatoxin producers in insects and fish from Zambia.

The current study sought to (i) quantify aflatoxins in insects and fish from markets in Zambia, (ii) characterize communities of *Aspergillus* section *Flavi* on insects and fish, and (iii) assess the capacity of insects and fish from Zambia to support growth and aflatoxin production by the observed *Aspergillus* section *Flavi*.

MATERIALS AND METHODS

Sampling. Dried caterpillar larvae (97 samples), termites (4 samples), and fish (66 samples) were obtained from markets in nine districts in Zambia: Mansa, Serenje, Lusaka, Kaoma, Kapiri Mposhi, Mazabuka, Choma, Livingstone, and Sesheke. Three morphologically distinct caterpillars were collected and later identified as *Gonimbrasia zambesina* (Fig. 1a), *Nephele* sp. (Fig. 1b), and *Gynanis maja* (Fig. 1c). A quarter of the *Gonimbrasia zambesina* samples were later found to also contain up to five *Gonimbrasia belina* individuals per kilogram (Fig. 1d). The fish were in genera *Oreochromis, Petrocephalus,* and *Limnothrissa* (Fig. 2), and the termites were all *M. falciger*. Where it was possible, five samples (350 to 500 g each) of each species were obtained from each market, with at least three markets from each district. All samples were dried in a forced air oven (40°C) to 5 to 8% water content at the University of Zambia to prevent fungal growth during transportation and then sealed in plastic bags to prevent rehydration. The insects and fish were imported to the U.S. Department of Agriculture (USDA), Agricultural Research Laboratory in the School of Plant Sciences, University of Arizona, under permit P526P-12-00853 awarded to P.J.C. by the Animal and Plant Health Inspection Service of the USDA.

Species assignment for caterpillars. To correctly assign species to caterpillars in the current study, cytochrome *c* oxidase subunit 1 (COI), a sequence widely used in insect and lepidopteran taxonomy *(15, 16, 36),* was amplified from caterpillar genomic DNA, sequenced, and compared with GenBank sequences of previously described species *(46)* by phylogenetic analysis. DNA was isolated from individual dried caterpillars according an *Aspergillus* spore-extraction protocol *(2),* with modifications. In brief, four caterpillars from each of three species were washed in 80% ethanol with 0.1% Tween, rinsed in sterile distilled deionized water, and left to dry. Ground insect samples were placed into 500 μL of lysis buffer (30 mmol L^{-1} Tris, 10 mmol L^{-1} EDTA, 1% sodium dodecyl sulfate, pH 8.0) and incubated in a Thermomixer 5436 shaker (Eppendorf, Inc., Hamburg, Germany) for 1 h at 60°C and 800 rpm. After removing cell fragments by centrifugation, DNA was precipitated using ammonium acetate and ethanol *(37)* and resuspended in 25 μL of sterile water. Twenty microliters of phenol–chloroform–isoamyl alcohol (25:24:1; 10 mM Tris, pH 8.0, 1 mM EDTA) was added to purify the isolated DNA, shaken for 1 min, and centrifuged. The supernatant was transferred to a fresh tube, and ammonium acetate and ethanol were used to precipitate DNA *(37)*. DNA was quantified with a spectrophotometer (model ND-1000, NanoDrop Technologies, Wilmington, DE) and diluted to a final concentration of 5 to 10 ng/μL before PCR.

The 658-bp COI fragment was amplified using primer pair LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) *(13, 16)*. PCR reactions were performed in 20-μL reactions by using 2 μL of genomic DNA and a PCR premix (AccuPower HotStart, Bioneer Pacific, Kew East, Victoria, Australia) with one cycle of 1 min at 94°C; five cycles of 1 min at 94°C, 1.5 min at 45°C, and 1.5 min at 72°C; 35 cycles of 1 min at 94°C, 1.5 min at 50°C, and 1 min at 72°C; and a final cycle of 5 min at 72°C *(16)*.

To correctly assign caterpillars in the current study to species, a BLAST search in GenBank was used for the COI sequence, and the three top matches were included in Bayesian analyses using MrBayes 3.2.6 *(18)*. Reference sequences obtained from GenBank were for the saturniids *Gynanisa maja* subsp. *terrali* (accession no. KF491774), *Gonimbrasia ertli* (accession no. HQ574035), *Gonimbrasia epimethea* (accession no. HQ574036), *Gonimbrasia longicaudata* (accession no. HQ573883), *Lobobunaea goodii* (accession no. HQ573808), *Nudaurelia jamesoni* (accession no. HQ574076), *Bunaea alcinoe* (accession no. HQ574067), and *Athletes albicans* (accession no. HQ574077) and the sphingids *Nephele comma* (accession no. FJ485749 and JN678292), *Nephele discifera* (accession no. JN678294), *Nephele lannini* (accession no. JN678298), *Nephele monostigma* (accession no. JN678300), and *Nephele subvaria* (accession no. JN678305) (*46*). *Pyralis farinalis,* in the family Pyralidae, which is a sister taxon to both Saturniidae and Sphingidae *(16),* was used to root the tree. Bayesian analyses were conducted with 10 million generations, and branches with less than 95% posterior probability were collapsed. Trees were visualized with FigTree 1.4.3 (http://tree.bio.ed.ac.uk/).

Aflatoxin quantification in ground insects and fish. Total aflatoxins were quantified (Table 1) with a lateral flow immunochromatographic assay (Reveal Q+ for Aflatoxin, Neogen Corporation, Lansing, MI) approved by Grain Inspection, Packers and Stockyards Administration; modifications to the manufacturer's instructions recommended by the administration were followed. In brief, each insect or fish sample (350 to 500 g) was ground with a knife mill (Retsch GM200, Retsch GmbH, Haan, Germany) to pass 75% of the ground material through a 20-mesh sieve, mixed thoroughly, and a 50-g subsample was blended with 250 mL of 65% ethanol. Aflatoxin content was determined according to the manufacturer's instructions. Because this aflatoxin quantification technique was not designed for insects and fish, the readings obtained were corrected using percent recovery data from spike and recovery assays. A briefly ground insect sample (5 g) with no detectable aflatoxin was spiked to 100 μg/kg total aflatoxin

FIGURE 1. *Caterpillars used in the current study as collected from markets in Zambia: (a)* Gonimbrasia zambesina, *(b)* Nephele *sp. (identified in current study), (c)* Gynanisa maja, *and (d)* Gonimbrasia belina. *Identification based on morphology (a, c, and d) and phylogenetics (a through d).*

FIGURE 2. *Fish used in the current study as collected from the markets in Zambia: (a, c)* Petrocephalus, *(b)* Oreochromis, *and (d, e)* Limnothrissa. *Identification based on morphology.*

TABLE 1. *Aflatoxin before and after incubation (31°C, 100% relative humidity, 7 days) of insects and fish from Zambia*[a]

Genus	No. of samples	Aflatoxin (μg/kg)		% samples > 9.9 μg/kg[b]	Aflatoxin after incubation (μg/kg)		
		Mean	Range		B_1	G_1	Total
Gynanisa	49	11 BC	2.9–24.4	40.6	214	15	229 B*
Gonimbrasia	44	12 B	3.4–25.1	54.8	3,197	4,832	8102 A*
Nephele	4	5 CD	4.3–6.1	0	3,315	2,370	6,187 A*
Macrotermes	4	24 A	16–36.8	100	NA[c]	NA	NA
Petrocephalus	25	9 CD	ND–20.4	40	9	ND	9 C
Limnothrissa	35	5 D	ND–17.2	15.8	10	ND	10 C
Oreochromis	6	ND[c] E	ND	0	23	ND	23 C*

[a] Values are the means of eight replicates. Total aflatoxin includes B_1, G_1, B_2, and G_2. Asterisks indicate significant differences in aflatoxin content before and after incubation by paired *t* test ($P < 0.05$). All data were log transformed before analyses, but actual means are presented. Means followed by the same letter in each column are not significantly different (at $P = 0.05$) by Tukey-Kramer's HSD test.
[b] Samples above 9.9 μg/kg are considered unsafe for human consumption in Zambia.
[c] NA, not applicable; ND, nondetected (limit of detection = 2 μg/kg).

by using an aflatoxin standard (in methanol; Supelco, Bellefonte, PA). Total aflatoxin was extracted and quantified as described above. Spike and recovery were performed in five replicates. Recovery rates were estimated using the following equation: % recovery = (aflatoxin concentration measured/spiked concentration) × 100.

The precision of the analytical method was expressed as relative standard deviation of replicated results. Recovery rates ranged from 40 to 60%. The limit of detection for Reveal Q+ for aflatoxin is 2 μg/kg, and the range of detection is 2 to 150 μg/kg.

Isolation and identification of fungi from insects and fish. Insect and fish samples were ground in a knife mill (Grindomix GM200, Retsch GmbH) to pass a no. 12 sieve, and then samples were homogenized. Fungi were isolated from ground insect and fish material by using a dilution plate technique on modified rose bengal agar *(6)*. In brief, ground insect and fish material (0.1 to 10 g) was shaken for 20 min at 100 rpm in 50 mL of sterile distilled water on a reciprocal shaker (KS-501, IKA Works Inc., Wilmington, NC). Dilution plating of the suspension was performed on modified rose bengal agar in triplicate. Plates were incubated for 3 days at 31°C in the dark, and up to eight colonies of *Aspergillus* section *Flavi* were transferred to 5-2 agar (5% V8 juice and 2% agar, pH 5.2). Fungi were stored in sterile water (2 mL) as plugs of sporulating culture after incubation for 7 days at 31°C *(4)*. Isolations were performed at least twice from each sample. *Aspergillus* species and strains were identified (Table 2) using macroscopic and microscopic characteristics *(5, 6, 22, 34)*.

Determining potential for aflatoxin formation after market. To determine the potential for aflatoxin concentrations to increase in market insects and fish during handling and storage, a technique previously applied to maize and groundnut, a simulated poor storage assay (*20*), was conducted. In brief, uninoculated caterpillar ($n = 10$) and fish ($n = 10$) market samples (Table 1) were thoroughly hand mixed, and then 10 g was placed onto metal sieves (10 cm in diameter) that were in sealed plastic boxes containing a moist sponge (4 by 4 by 4 cm) and incubated at 31°C for 7 days. After incubation, samples (Fig. 3) were ground in a blender (Waring 7012S, Waring, Torrington, CT) containing 50 mL of 70% methanol at maximum speed for 20 s. The slurry was allowed to settle (20 min), and 4 μL of the supernatant was spotted directly onto thin-layer chromatography plates (Silica gel 60, EMD, Darmstadt, Germany) adjacent to aflatoxin standards (Aflatoxin Mix Kit-M, Supelco) containing known quantities of aflatoxins B_1, B_2, G_1, and G_2. Plates were developed in ethyl ether–methanol–water (96:3:1) and then air dried after which aflatoxins were visualized under UV light (365 nm). Aflatoxins were quantified directly on thin-layer chromatography plates by using a scanning densitometer (TLC Scanner 3, Camag Scientific Inc., Wilmington, NC) running winCATS 1.4.2 (Camag Scientific Inc.). The limit of detection and range of detection for the aflatoxin quantification technique used was 5 μg/kg and 5 to 500,000 μg/kg, respectively. Recovery rate for this technique was not determined.

Suitability of insects and fish as substrates for growth and aflatoxin production by toxigenic *Aspergillus* section *Flavi*. To evaluate the ability of caterpillars and fish from markets to support growth and aflatoxin contamination, inoculation tests were

TABLE 2. *Distribution of fungi of* Aspergillus *section* Flavi *on edible insects and fish from Zambia*[a]

Genus	No. of samples	%L[b]	%S	%P	%T	CFU/g
Gynanisa	49	36 B XY	13 A Y	44 A X	7 AB Y	31 A
Gonimbrasia	44	55 AB X	13 A Y	25 AB XY	7 AB Y	45 A
Nephele	4	66 AB X	16 A Y	18 B Y	0 B Y	22 A
Macrotermes	4	68 AB X	0 A Z	0 B Z	32 A Y	6 A
Petrocephalus	25	88 A X	4 A Y	5 B Y	3 B Y	28 A
Limnothrissa	35	85 A X	10 A Y	3 B Y	2 B Y	19 A
Oreochromis	6	92 A X	0 A Y	0 B Y	8 AB Y	59 A

[a] Values followed by the same letter in each column (A, B, C) or row (X, Y, Z) do not differ by Tukey's HSD test ($\alpha = 0.05$). Percent data were arcsine transformed, and CFU per gram data were log transformed before analyses, but actual means are presented here.
[b] L, S, P, and T represent the *A. flavus* L morphotype, S morphotype fungi, *A. parasiticus,* and *A. tamarii,* respectively.

FIGURE 3. *Caterpillars exiting incubation during the simulated poor storage assay tests with visually evident fungal growth on uninoculated, incubated caterpillars.*

performed with known aflatoxigenic *Aspergillus* section *Flavi* genotypes. In brief, four isolates representing *A. flavus* L morphotype strain AF13 (ATCC 96044, SRRC 1273), *A. flavus* S morphotype strain AF70 (ATCC MYA384), *A. parasiticus* (NRRL2999), and an unnamed taxon S_{BG} (A-11612; Tables 3 and 4) were inoculated onto caterpillars and fish (10 g in a 250-mL Erlenmeyer flask) previously autoclaved for 20 min, cooled to room temperature, and moisture adjusted to 30%. One million freshly harvested spores from 7-day-old cultures of each isolate were used in the inoculations. The inoculated insects and fish were incubated for 7 days at 31°C and 100% relative humidity to allow fungal growth and aflatoxin production and quantity of *Aspergillus* section *Flavi* CFU (primarily spores) produced per gram of substrate to be determined. After incubation, 100 mL of sterile distilled deionized water with 0.1% Tween was added to each culture and shaken at 650 rpm on a mini orbital shaker (Troemner LLC, Thorofare, NJ) for 10 min. The resulting spore suspension was subjected to a 10-fold dilution series and plated onto rose bengal agar in four replicates. The amount of spores produced per gram of substrate was expressed on a dry weight basis. After incubation, the sample cultures (Fig. 4) were blended in 50 mL of

TABLE 3. *Growth of four aflatoxin-producing fungi on edible caterpillars and fish from Zambia*[a]

Genus	Growth of four aflatoxin producers (log CFU/g dry wt basis)				Avg (log CFU/g)
	A. parasiticus	*A. flavus* S	*A. flavus* L	Unnamed S_{BG}	
Gynanisa	6.89 B Y	4.88 A Z	7.69 C X	9.00 A W	7.12 B
Gonibrasia	6.41 C X	4.27 B Y	11.94 B W	6.78 B X	7.35 A
Nephele	8.92 A Y	4.87 A Z	12.22 A W	9.52 A X	8.88 A
Oreochromis	3.71 D Y	3.83 C Y	6.17 E X	2.05 E Z	3.94 C
Petrocephalus	NA[b]	NA	NA	2.73 D	
Limnothrissa	3.86 D Z	4.83 A X	7.00 D W	4.29 C Y	5.00 C
Avg	5.96 Y	4.54 Z	9.00 X	6.33 Y	

[a] Values are means of four replicates. Those followed by the same letter in each column (A, B, C) or row (X, Y, Z) are not significantly different (at $P = 0.05$) by Tukey-Kramer's HSD test.

[b] NA, not applicable (tested).

TABLE 4. *Aflatoxin production by four aflatoxin producers on edible caterpillars from Zambia*[a]

Caterpillar genus	Aflatoxin production by four toxigenic aspergilli (μg/kg)				Avg (μg/kg)
	A. flavus L	*A. flavus* S	*A. parasiticus*	Unnamed S_{BG}	
Gynanisa	62,800 X AB	48,000 X B	14,800 X A	65,500 X A	47,800 B
Gonibrasia	21,300 XY B	68,200 X B	8,600 Y A	15,200 XY A	28,300 B
Nephele	106,800 XY A	263,800 W A	15,600 Z A	72,900 Y A	114,800 A
Avg	63,700 X	126,700 W	13,000 Y	51,200 X	63,600

[a] Means followed by the same letter in each column (A, B, C) or row (W, X, Y, Z) are not significantly different (at $P = 0.05$) by Tukey-Kramer's HSD test. All data were log transformed before analyses, but actual means are presented.

70% methanol, and aflatoxins were quantified with thin-layer chromatography as described above.

Data analysis. Aflatoxin content in fish and insects purchased directly from the markets and aflatoxin produced in laboratory inoculation experiments were measured in micrograms per kilogram. Total quantities of section *Flavi* in market samples and after inoculation, incubation, or both were calculated as CFU per gram of substrate. Community composition of section *Flavi* was described as a percentage of *A. flavus* L strain morphotype *(5),* undelineated S strain morphotype *(33),* and *A. parasiticus* recovered from each sample. Aflatoxins before and after incubation were compared using a paired *t* test and multiple means (aflatoxins, CFU per gram, and percent) were compared using analysis of variance (ANOVA) general linear models and Tukey's honestly significant difference (HSD) test as implemented in JMP 11.1.1 (SAS Institute Inc., Cary, NC). Data were tested for normality and, if required, log transformed (aflatoxin and CFU data) to normalize distributions before analyses. Percent data were arcsine transformed before analyses; however, actual means are presented for clarity. All tests were performed at $\alpha = 0.05$.

RESULTS

Species assignment for caterpillars. To verify species assignment, caterpillars from Zambia were compared with known species through phylogenetic analyses of 658 bp of COI. Three groups of caterpillars were resolved, with all caterpillars morphologically assignable to *Gynanisa maja* grouping with reference *Gynanisa maja* (NCBI accession no. KF491774) in group 3 (Fig. 5). Caterpillars assigned to *Gonimbrasia zambesina* based on morphology grouped with, but remain distinct from, other *Gonimbrasia* (*G. ertli,* NCBI accession no. HQ574035; *G. epimethea,* NCBI accession no. HQ574036; *G. longicaudata,* NCBI accession no. HQ573883; Fig. 5, group 2). Reference sequences were not found for either *Gonimbrasia zambesina* or *Gonimbrasia belina*. The third morphological species purchased in Zambian markets was found only in Kaoma and grouped with members of the genus *Nephele* (*N. comma,* NCBI accession no. FJ485749 and JN678292; *N. discifera,* accession no. NCBI JN678300; *N. subvaria,* NCBI accession no. JN678305; Fig. 5, group 1).

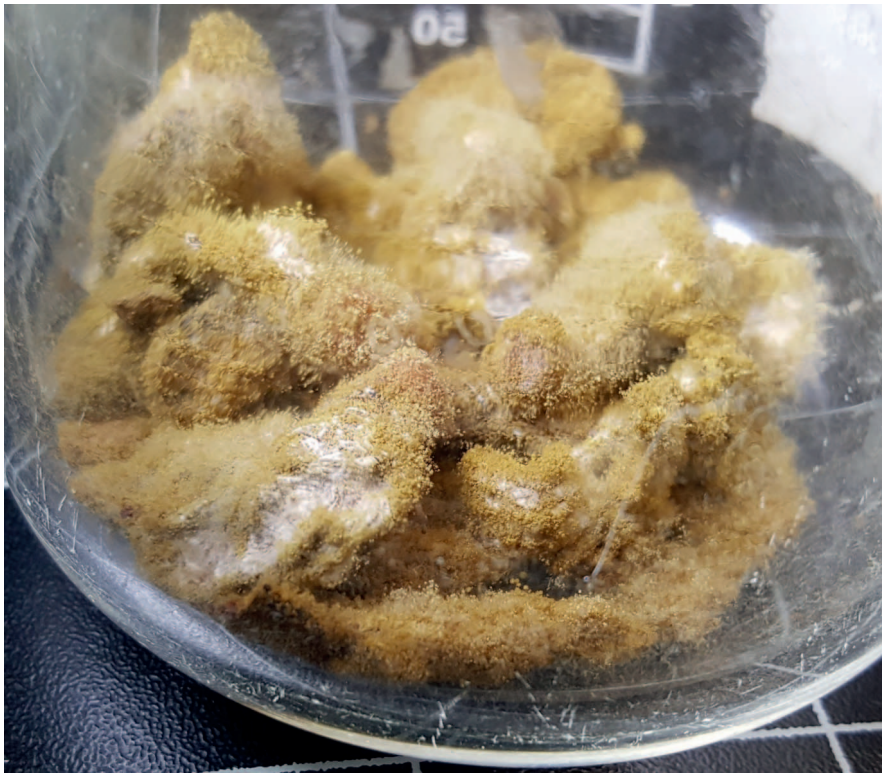

FIGURE 4. *Fungal growth on caterpillars inoculated with* A. parasiticus.

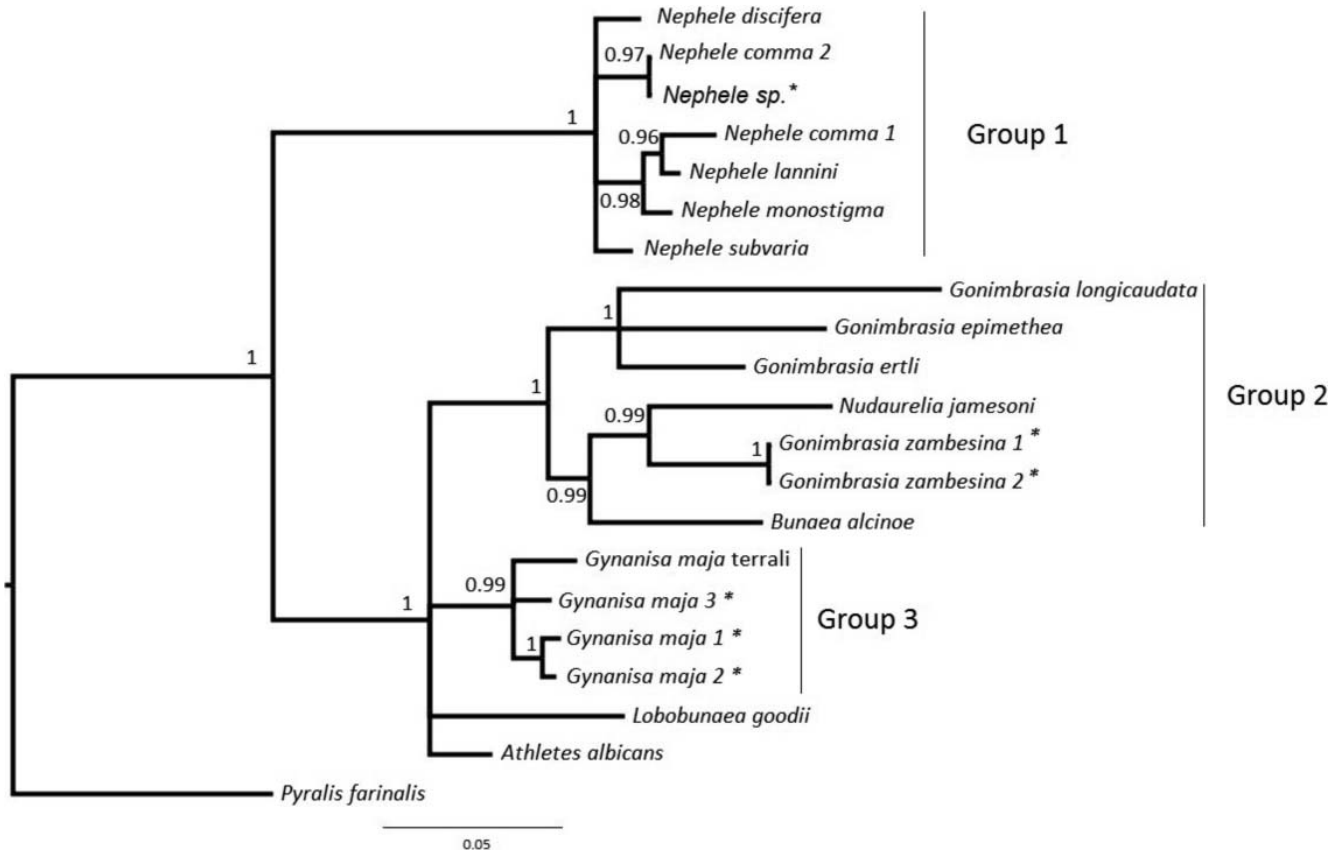

FIGURE 5. *Phylogenetic relationships among caterpillars purchased in markets in Zambia and known species. Bayesian tree; sequences for purchased species developed in the current study; sequences for reference taxa from North America and Costa Rica. Tree is based on cytochrome* c *oxidase subunit 1 (COI, 658 bp). Support values above nodes indicate posterior probabilities. Group assignments: group 1,* Nephele*; group 2,* Gonimbrasia*; group 3,* Gynanisa*. Asterisks indicate taxa from Zambia.*

Aflatoxin in insects and fish. There were significant differences (ANOVA: $F_{6,98} = 13.3965$; $P < 0.001$) in total aflatoxin concentration in market samples of various species of caterpillars and fish. The highest average aflatoxin concentration (24 μg/kg) was in termites (*M. falciger*). Only the members of the fish genus *Oreochromis* had no detectable aflatoxins (Table 1). Percent samples exceeding 10 μg/kg (Zambian regulatory limit for aflatoxin in food) was 100% for termites; 54.8 and 40.6% for the caterpillars *Gonimbrasia zambesina* and *Gynanisa maja,* respectively; and 15.8% for members of the fish genus *Limnothrissa* (Table 1). Although none of the *Nephele* sp. (identified in current study; Fig. 5) samples were above the Zambian regulatory limit, all were above the European limit of 4 μg/kg.

Potential for aflatoxin formation after market. During the simulated poor storage assays, increases in aflatoxin content of several orders of magnitude were observed in several insect and fish samples. Increases to unacceptable concentrations were observed even in samples that initially had acceptable aflatoxin levels (Table 1). Total aflatoxins increased at least 20-fold (on average, from 11 to 229 μg/kg; paired *t* test, $P < 0.001$) in *Gynanisa maja,* 600-fold (from 12 to 8,102 μg/kg; paired *t* test, $P < 0.001$) in *Gonimbrasia zambesina,* 1,000-fold (from 5 to 6,187 μg/kg; paired *t* test, $P < 0.001$) in the *Nephele* sp., and from nondetectable levels to 23 μg/kg (paired *t* test, $P < 0.05$) in *Oreochromis* (Table 1). No significant increases were observed in the fish genera *Petrocephalus* and *Limnothrissa*.

Fungi from insects and fish. *Aspergillus parasiticus, A. flavus* L morphotype, fungi with S morphology, and *A. tamarii* were recovered (Table 2). The *Aspergillus flavus* L morphotype was the most common *Aspergillus* section *Flavi* species associated with each of the insect and fish species, with the exception of *Gynanisa maja* for which *A. parasiticus* was the most frequent. There were significant differences (ANOVA: $F_{6,43} = 5.2317$; $P < 0.001$; Table 2) in *A. flavus* L morphotype frequencies on insects and fish, with the highest occurrence on *Oreochromis* (92%) and the least on *Gynanisa maja* (36%). Although frequency of *Aspergillus* section *Flavi* with S morphology was similar (8% ± 8%) among all the fish and insects (ANOVA: $F_{6,43} = 0.6835$; $P = 0.6638$; Table 2), the frequencies of *A. parasiticus* differed (ANOVA: $F_{6,43} = 4.6609$; $P = 0.001$; Table 2), with the highest frequency (44%) on the caterpillar *Gynanisa maja* and none detected on *Oreochromis* and *M. falciger*. Overall quantities (CFU per gram) of *Aspergillus* section *Flavi* were similar (30 ± 29 CFU/g) for all species (ANOVA: $F_{6,43} = 1.4889$; $P = 0.2056$; Table 2).

Suitability of insects and fish as substrate for growth by toxigenic *Aspergillus* section *Flavi*. All insects and fish tested supported growth of all three *Aspergillus* species evaluated (Table 3 and Fig. 4). Species differed in ability to support growth by aflatoxin producers (ANOVA: $F_{5,17} = 17.7761$; $P < 0.001$), with the most growth (Tukey's HSD test, $P < 0.05$), on average, on *Nephele* sp. (4.1×10^{15} CFU/g) and the least on *Oreochromis* (4.2×10^{9} CFU/g; Table 4). Average growth across all insects and fish for all fungi were also different ($F_{5,17} = 17.6324$; $P < 0.001$). There were significant differences among species of insects and fish (ANOVA: $F_{4,20} = 3541.340$; $P < 0.001$) in support of *A. parasiticus* growth with the most propagules produced on *Nephele* sp. (8.4×10^{12} CFU/g) and the least on *Oreochromis* (5.2×10^{7} CFU/g; Tukey's HSD test, $P < 0.05$; Table 3). Differences (ANOVA: $F_{4,20} = 267.1297$; $P < 0.001$) were also observed in support of growth of the *A. flavus* S morphotype AF70 (ATCC MYA384) with the most growth on *Gynanisa maja* (7.8×10^{8} CFU/g), *Nephele* sp. (7.4×10^{8} CFU/g), and *Limnothrissa* (6.8×10^{8} CFU/g) followed by *Gonimbrasia zambesina* (1.9×10^{8} CFU/g) and *Oreochromis* (6.9×10^{7} CFU/g) (Table 3). The other *A. flavus* (AF13; ATCC 96044, SRRC 1273, L morphotype) had much higher growth on *Nephele* sp. (1.7×10^{16} CFU/g) than on any other insect or fish (Tukey's HSD test, $P < 0.05$). For the unnamed taxon S_{BG} (A-11612) as with the other three fungi, *Oreochromis* supported the least growth.

Aflatoxin production on insects and fish. Each of the three caterpillar species supported production of more than 28,000 μg/kg total aflatoxins (Table 4). The *A. flavus* S morphotype produced the highest concentrations of aflatoxin (126,700 μg/kg), with the most aflatoxins forming on *Nephele* sp. (263,800 μg/kg) (Table 4). Production of aflatoxins differed among caterpillars for both the *A. flavus* L morphotype (ANOVA: $F_{2,8} = 8.0041$; $P = 0.0203$) and the *A. flavus* S morphotype (ANOVA: $F_{2,8} = 36.6697$; $P < 0.001$). No differences were observed in aflatoxin production by either *A. parasiticus* (ANOVA: $F_{2,8} = 0.5353$; $P = 0.6111$) or the unnamed S_{BG} (ANOVA: $F_{2,8} = 3.0788$; $P = 0.1202$) on fish and insects.

DISCUSSION

Aflatoxin in insects and fish. Aflatoxins are a danger to human health, livestock productivity, and trade *(14, 24, 33, 35, 41, 42, 45, 47)*. Deaths from consumption of highly contaminated food in Kenya *(23, 24, 33)* and Tanzania and increasing mycotoxin safety concerns in edible insects *(31, 43)* have led to the need to evaluate the safety of foods originating from insects. In the current study, aflatoxins were detected in almost all fish and insects evaluated, with the termite *M. falciger* and the caterpillars *Gynanisa maja* and *Gonimbrasia zambesina* having average levels above those allowed for food in Zambia (Table 1). Aflatoxin levels in caterpillars in the current study were different from those previously reported, where average aflatoxins in many locations exceeded 20 μg/kg *(31)*. These differences may result from differences in species examined, differences in environmental conditions to which the insects were subjected during processing and storage *(8, 20)*, or differences in *Aspergillus* section *Flavi* community compositions *(21, 34)*. In contrast, average aflatoxin concentrations in fish in the current study were similar to those previously reported on *Gadus morhua, Katsuworus pelamis, Pseudotolithus typhus, Dasyatis margarita, Arius hendeloti, Ethalmosa fimbriata, Triuchurius trichurius, Carchanas faunis, Pentanemis qumquarius, Cynoglossus browni,* and *Drepane africana (1, 19)*. However, the proportion of fish with aflatoxins ≥10 μg/kg in *Petrocephalus* (40%) and *Limnothrissa* (15.8%) in the current study is still a reason for concern, given their importance in human diets (Fig. 6). None of the *Oreochromis* fish had detectable aflatoxins. Given that *Oreochromis* is processed using techniques and environments similar to those of *Petrocephalus* and *Limnothrissa,* the absence of aflatoxins in *Oreochromis* suggests it is either not a suitable substrate for aflatoxin production or may not contain high enough proportions of aflatoxigenic fungi *(8, 20, 34)*. Results indicate that aflatoxins are common in marketed dried insects, but not marketed dried fish.

Fungi from insects and fish. To assess the potential for food to become contaminated with aflatoxins, frequencies of aflatoxin-producing fungi must be considered *(30, 33)*. *Aspergillus flavus* L morphotype dominated most insects and fish, although appreciable amounts of the consistently aflatoxigenic *A. parasiticus* and S morphology fungi were also found on the three caterpillar species (Table 2). Previous studies of fungi on edible caterpillars and fish *(19, 31)* have listed *A. flavus* L as the only *Aspergillus* section *Flavi* on caterpillars. The current study reveals that additional *Aspergillus* section *Flavi* species can occur on caterpillars and that the etiology of aflatoxin contamination of these valuable foods could be complex. The L morphotype of *A. flavus* is associated with high variability in aflatoxin production, with both highly aflatoxigenic and atoxigenic genotypes *(5)*. In Zambia, high prevalence of the L morphotype of *A. flavus* has been associated with low aflatoxins in maize and groundnuts *(20)*. High L strain incidence may partially explain the low levels of aflatoxins observed in fish (Table 1), particularly *Oreochromis,* where the L morphotype was as high as 92% and no S morphotype or *A. parasiticus* was detected (Table 2). Similarly, *Nephele* sp. had lower amounts of aflatoxins compared with the other two caterpillar species (Table 1), possibly because the former had higher incidences of the L strain morphotype than the other caterpillar genera. In addition, *Nephele* also had lower proportions of *A. parasiticus* and fungi with S morphology than the other two caterpillar genera (Table 2). *A. parasiticus* and fungi with S morphology are almost always highly aflatoxigenic, and frequencies of S morphotype fungi as low as 13%, as were observed with caterpillars in the current study, can cause high aflatoxins levels *(9)*. It is expected that under poor storage, aflatoxins might still increase in *Nephele* as both the average aflatoxin-producing potential of the fungal community and the extent of growth both contribute *(8, 20)*.

FIGURE 6. *Insects and fish in markets in Zambia:* Gynanisa *(a),* Gonimbrasia *(b),* Oreochromis *(c), and* Limnothrissa *(d).*

Potential for aflatoxin formation after market. When aflatoxin producers are present in food with low aflatoxins, as is the case in the current study, aflatoxin increases may occur during processing, transport, or poor storage. A method for quantifying the risk of aflatoxin increase *(8, 20)* was applied in the current study. Aflatoxins increased by at least 20-fold in the caterpillars and by 4-fold in *Oreochromis* after simulated poor storage assays (Table 1). This suggests that even though aflatoxins in *Nephele* from markets in Zambia were present at permissible levels initially, poor storage could create environments conducive for aflatoxin production by the aflatoxin-producing fungi already colonizing the insects at purchase (Table 2). This could result, as demonstrated in the current study (Table 1), in *Nephele* with aflatoxin concentrations unsuitable for human consumption (Table 1). Although aflatoxin levels in incubated *Oreochromis* rose during simulated poor storage assays (Table 1), the increase was much lower than what would be expected where all environmental conditions are conducive for aflatoxin production and highly aflatoxigenic fungi are present. This low increase in aflatoxins in *Oreochromis* suggests that the colonizing *A. flavus* L morphotype fungi may have included significant numbers of atoxigenic genotypes that interfere with the contamination process *(30)*. However, because the termites had both high aflatoxins in the market and high incidences of the *A. flavus* L strain, either the colonizing fungal populations differ in incidence of atoxigenic genotypes or other factors, such as nutritional composition of *Oreochromis (29),* might have played roles in reduced aflatoxin concentrations in *Oreochromis* both at the market and after incubation.

Suitability of insects and fish as substrate for growth and aflatoxin production by aflatoxigenic *Aspergillus* section *Flavi*. Growth and quantities of aflatoxins produced by *A. flavus, A. parasiticus,* and the phylogenetically diverse fungi with S morphology can differ among fungal isolates and between different substrates *(29)*. To assess the suitability of fish and insects as hosts for growth and aflatoxin production, representative genotypes from the fungal groups mentioned in the preceding sentence were inoculated onto sterile insects and fish. All species tested support growth by aflatoxigenic fungi (Table 3). However, caterpillars were better substrates for growth of aflatoxigenic fungi than fish (Table 3). In nature, members of the genus *Aspergillus* are known to be entomopathogenic *(3, 12)* and may have evolved to effectively use diverse insects as food sources. This may explain why more growth occurred on caterpillars than on fish (Table 3). In addition, caterpillars supported accumulation of high concentrations of aflatoxins (>60,000 μg/kg). Aspergilli have been reported to infect and kill insect hosts by producing aflatoxins *(12)*; therefore, it is not surprising to see aflatoxin producers growing well and producing significant quantities of aflatoxins on food insects from Zambia in the current study. Aflatoxin producers have been shown to be capable of contaminating these foods, with concentrations of aflatoxins being many times the legally allowed levels. Although consumers will not cook and eat foods with profuse fungal growth as shown in Figures 3 and 4, the current study demonstrates that insects support growth and aflatoxin production by some aflatoxin producers; as such, proper handling of the foods is needed.

Species assignment for caterpillars. Lepidopteran larvae are normally harvested for food after the third or fourth molt and then gutted, dried, and cooked before consumption *(27)*. During these processing steps, features diagnostic of each species may be lost. In addition, caterpillars not yet described may be consumed in Zambia *(25)*. To ensure correct species assignment, phylogenies based on 658 bp of COI were reconstructed to compare the species sampled in markets to reference taxa for which sequences had been deposited in NCBI databases. Based on this analysis, at least three species of edible caterpillars were present in our collection: *Gynanisa maja* (Fig. 5, group 3), *Gonimbrasia* (Fig. 5, group 2), and a *Nephele* sp. closely related to *N. comma* (Fig. 5, group 1). Owing to paucity of

lepidopteran COI sequences in GenBank, larvae from the market initially identified based on morphology as *Gonimbrasia zambesina* and *Gonimbrasia belina* could only be confirmed to the genus level (Fig. 5).

In summary, aflatoxins and aflatoxin-producing fungi are common on insects and fish in Zambia. The presence of aflatoxigenic fungi in these foods poses a risk of increased aflatoxin contamination when they are poorly stored. Fish and insects, particularly caterpillars, are suitable substrates for aflatoxin biosynthesis. Aflatoxin mitigation measures have targeted major agricultural products including cereals, peanut, tree-nuts, and crop by-products. However, it is evident from the current study that insects and fish could also be problematic routes for exposure to aflatoxins. Aflatoxin mitigation measures should take into consideration exposure originating from beyond agricultural commodities and include dried, edible insects and fish (Fig. 6) so that foods with aflatoxins of 10 μg/kg and above are excluded from the food chain in Zambia.

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