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The Effect of Symbioses Between Mold Mites (Acaridae: Tyrophagus putrescentiae) and Aspergillus flavus on their Respective Populations in Stored Maize.

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The Effect of Symbioses Between Mold Mites (Acaridae: *Tyrophagus putrescentiae*) and *Aspergillus flavus* on their Respective Populations in Stored Maize.

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Abstract

Zea mays is the most commonly grown grain in the world and is used for animal feed, human consumption, and the creation of other products such as ethanol and bioplastics. Contamination of stored maize with stored grain invaders can lead to loss of revenue, reduced food availability, and potential health complications. Two such storage invaders; the mold mite *Tyrophagus putrescentiae* and the fungus *Aspergillus flavus* can work as symbionts within the grain storage system. To determine resulting population growth from this symbiosis, maize was inoculated with three treatments: *A. flavus* with no mites, *T. putrescentiae* with no fungus, and both organisms together. Treated maize samples were kept under stable humidity and temperature conditions and sampled over a 42-day period. Population numbers of *A. flavus* and *T. putrescentiae* were determined through qPCR and counting software, respectively. Treatments containing both organisms showed evidence of *A. flavus* and *T. putrescentiae* populations cycling together with complimenting population increases and decline. Treatments with just *T. putrescentiae* or just *A. flavus* showed a linear population growth.

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Dedication

This thesis is dedicated to my family: the Cummins' and the Pionke's.

Table of Contents

List of Tables and Figures

Introduction

*Zea mays (L).*is the most commonly grown grain in the world with an estimated 44,600 million bushels grown this past year (USDA, January 2021). Maize is a large component in animal feed as well as a key ingredient in the production of ethanol, starch, syrups, bioplastics, and a favorite for general human consumption.

Storage conditions for maize must be monitored to prevent degradation and fungal growth. Luckily, modeling the heat and mass transfer maize undergoes when drying has allowed for the development of safe storage recommendations (Coradi et al. 2020), representing the longest period of time that grain can be stored without deterioration (Sadaka et al. 2016). The recommendations are dependent on temperature and humidity in addition to hybrid of corn used and are often given in the form of safe moisture content (MC) percentage for length of time stored (days). The moisture content of any given grain, under relatively fixed circumstances, will stabilize with its environmental conditions to the point where it will neither lose nor absorb moisture. This is called the Equilibrium Moisture Content (EMC) and can be calculated using through the use of set standards with the use of temperature and relative humidity. Higher moisture content can lead to increased biological activity, and conversely, excessively dried grain can lead to weight loss. When sold, a bushel of shelled corn weighing 56 lbs is at an assumed 15.5% moisture Content (Kelley & Capps. n.d.)

Improper implementation of these recommendations can result in grain deterioration. “Grain deterioration is usually expressed as percent of dry matter loss” with a 0.5% loss level being the declination of a U.S. grade level (Sadaka et al. 2016). Postharvest losses from handling, storage, and drying mishaps can make up 25-30% of the value produced (Coradi et al. 2020). These losses can be increased due to biological activity. The formation of localized hotspots through

respiration (glucose and oxygen reactions) creates a warm, moisture rich environment in which fungi, microbes, and arthropod invaders thrive and further exacerbate post-harvest degeneration.

Aspergillus flavus

The genus *Aspergillus* was described in 1729 by Pier Antonio Micheli because of the resemblance between the conidia of *Aspergillus* spp. and an aspergillum holy water sprinkler (CDC, 2006). *Aspergillus flavus* (Link, 1809) is a filamentous Ascomycete. Although often found as a soil saprophyte, this species is an opportunistic pathogen of crops and animal species including humans. The fungus often grows as a powdery clump, yellow-green to army green in color. In stored grains, the fungus causes storage rot and produces aflatoxins as well as other mycotoxins posing a major human health risk. The species is split into two groups: L and S based on sclerotia size and aflatoxin production (Amaike & Keller, 2011). Production of sclerotia is linked to cell density with sparce growth resulting in more sclerotia (Horowitz Brown et al. 2008).

The anamorph spreads through the dispersal of conidia (asexual reproductive spores) that are commonplace in the soil, air, and decomposing organic matter. Passive dispersal of conidia is often achieved through airflow but can also be transported by arthropod activity (Hubert et al. 2003, 2004; Van Asselt, 1999; Griffiths et al. 1999). The heterothallic sexual stage is defined as *Petromyces flavus* with ascospores developing within sclerotia and reproduction occurring when compatible vegetative groups are cultured around each other (Horn et. al 2009).

Once in grain stores, *Aspergillus* growth can be supported by equilibrium moisture content (EMC) as low as 13% in stored corn. Growth of *Aspergillus* in stored maize can result in decreased germinability and nutritional value, musty odors, discoloration, and contribute to

heating (Sauer, 1988; Magan et al. 2003). Additional concerns with *Aspergillus* growth are the production of mycotoxins, specifically aflatoxins, whose presence renders grain unusable. Other concerns include the potential for mycosis such as aspergillosis in immunocompromised individuals.

After *A. parasiticus* (Speare, 1912), *A. flavus* is the second most common cause of invasive aspergillosis causing around 10% of infections worldwide (Rudramurthy et al. 2019). The disease is defined as a hyphal infiltration into tissue of an individual and can be seen by histological examination. Broadly, aspergillosis can be seen in four categories: local invasive infection resulting from trauma or post-surgery infection, sub-acute or chronic infection of people with lung structure abnormalities, allergic disease, or infection of immunocompromised individuals which, often proves life threatening ((Rudramurthy et al. 2019). Mortality of invasive aspergillosis at 30 days in adult ICU patients is estimated to be 33.1% (Lanjewar, 2011; Baddley et al. 2013).

Aspergillosis most commonly affects the lungs, sinuses, and central nervous system (Rudramurthy et al. 2019; Zhang et al. 2019). Secondary aspergillosis infections after respiratory distress caused from the influenza virus has been well documented. Recently, similar secondary infections have popped up after severe infection from SARS-CoV-2 (Marr et al. 2021). Aspergillosis is often treated through fungicides, causing inhibition of sterol biosynthesis with the active ingredient used differing based on the causal agent of symptoms such as, triazole antifungals including Voriconazole (Marr et al. 2021; Rudramurthy et al. 2019; Zhang et al. 2019)

Mycotoxins of the aspergilli

Mycotoxins are classified as secondary metabolites produced by filamentous fungi that are harmful to vertebrates in low concentrations. These compounds cause a variety of disruptions in the vertebrate body including, but not limited to, immunosuppression, inhibition of protein synthesis and metabolic pathways, contact irritation, and carcinogenicity (Bennett & Klitch, 2003). Mycotoxicoses, a disease caused by mycotoxin exposure, can be classified as either chronic or acute depending on the length of exposure, with the greatest risk to humans and livestock being through "chronic dietary exposure" (Tai et al. 2020). Different mold species produce different mycotoxins with further division of the compounds based on the color of UV fluorescence and chromatographic mobility. However, not all mycotoxins are easily identified, and there is increasing data showing the presence of "masked mycotoxins" whose chemical structure has been altered by plant enzymes during infection, making them hard to detect or identify (Tai et al. 2020).

Aflatoxins are a kind of mycotoxin produced by *Aspergillus* molds. Although thought of as the main toxigenic compound produced in the genus, only about half of the aspergilli produce aflatoxins. A variety of other mycotoxins are synthesized instead of or in conjunction with aflatoxin e. g., citrinin, ochratoxins, cyclopiazonic acid, and aflatrem (Amaike and Keller, 2011).

Aflatoxins are known to be carcinogenic, hepatotoxic, and mutagenic, with potency depending on the strain produced (Hedayati et al., 2007). In fact, the combined growth of *A. flavus* with *A. parisiticus* in foodstuffs are estimated to cause anywhere from 4.8 to 28% of the world's most common liver cancer, hepatocellular carcinoma (Liu et al. 2012; Liu & Wu, 2010; Tai et al. 2020).

Although there are dozens of varieties, the four most common kinds of aflatoxins are B1, B2, G1, and G2 (Bennet & Klitch, 2003). Aflatoxin B1 is the most potent hepatocarcinogen found to date and subsequently the most studied. AFB1 and B2 have the ability to carry over from solid feed to milk in the form of hydroxylated metabolites known as aflatoxins M1 and M2 (Tai et al. 2020; Omar, 2016). Similarly to other aflatoxins, M1 and M2 are resistant to pasteurization, autoclaving, and other food processing procedures (Omar, 2016).

In 1969, the FDA set the action threshold for aflatoxins at 20ppb in all food for direct human consumption, as well as pet food and immature animal feed. Higher thresholds are in place for feed intended for finishing livestock. A variety of studies have also shown that AFB1 has phytotoxic properties as well, leading to a decrease in germinability of seed through plant cell alteration (Klich & Lee, 1982; Mclean, 1994; McLean et al. 1995).

Tyrophagus putrescentiae

Another common invader of stored grains are mites. Storage mites are secondary invaders after grains have been damaged by primary pests such as beetles and moths and after fungal deterioration. Because of this, storage mites have long been, until recently, considered a pest of low economic importance. The advent of "Zero Tolerance" policies for storage grain arthropods, the relative ineffectiveness of insect pest controls on mites, and the ability for bursts in population size have elevated their pest status (Malik et al. 2018). Mites can feed on the germ of the kernel, reducing germinability, vitamin b content, and commodity weight. Grain stores that have been fed on for extended periods of time lose their sheen and start to look dull, occasionally, acquiring a "minty" taste from lipid secretions (Malik et al. 2018). Solomon (1946) indicated the mites cannot feed on kernels with intact pericarp, however, the threshing and storing process often results in scarification sufficient for feeding to occur (Krantz, 1955).

Further damage to grain supplies results from mite activity leaving a buildup of "dust" consisting of dead mites, fungi, feces, eggs, and further broken-down kernels, causing the commodity to be considered tainted (Hughs, 1976; Hagstrum et al. 2012). Over time, grains may acquire a lemony scent emitted by the mite in large infestations (Malik et al. 2018).

One of the more prevalent species found in stored grains is *Tyrophagus putrescentiae* (Schrank,1781), an astigmatid mite found in the family Acaridae. First described by Franz von Paula Schrank in his 1781 book *Enumeratio insectorum Austriæ indigenorum*, he names the species *Acarus putrescentiae* referring to them as colorless, ovate, fetid smelling mites. His description is grouped with an aside about springtails (Shrank, 1781). Since this original description, the species has accumulated twenty-one synonym names (Solarz, 2012). This and related species *T. longior* (Gervais, 1844) are often referred to as the "mould mite" the "cosmopolitan mite" or misidentified as the "cheese mite" (*Tyrophagus casei*).

According to a redescription by Solarz's in 2012, *T. putrescentiae* is defined as having: A propodosomal shield with pigmented spots along the anterior margins, seta *sc x* long and widened towards end half bearing 5-8 pairs of pectinations. Setae d 1 distinctly longer than *c 1* and *d 2*, posterior margin on coxal plate II sinuous and narrowing sharply toward the end third. Tarsi I and II with solenidion with solenidion ω I on tarsi I widened. The male aedeagus is shaped with two deep curves. There are tarsal suckers on *Ta* IV (Solarz 2012).

After oviposition, the life cycle of *T. putrescentiae* consists of a larval, protonymphal, tritonymphal, and adult stages with an average lifespan of ~ three weeks depending on their environmental conditions. The mite lacks a hypopal stage and the exact mechanism of dispersal for the species is unknown, but it is speculated that movement of contaminated material such as foodstuffs and plant matter may contribute to the process (Hagstrum et al., 2012). This dispersal

is exacerbated by the release of a neryl formate alarm pheromone in response to overcrowding, unfavorable environmental conditions, or the depletion of food source (Kuwahara et al. 1979).

Historically, *T. putrescentiae* is thought to be a fungivore, but is highly polyphagous and known to feed from high protein and fat content foods including cheeses, meats, nematodes, plant detritus, microorganisms, yeasts, dermatophytes, storage fungi, and over 140 different seed commodities (Hagstrum, 2016). Occasionally, the species has been reported as predator of larval stages of *Lasioderma serricorne* (Fabricius, 1792) and *Diabrotica undecimpunctata* (Mannerheim, 1843) eggs (Papadopoulou, 2006; Brust & House. 1988). This polyphagous feeding behavior makes human encounters common due to their tendency to inhabit human environments such as "household pantries and agricultural spaces like farms and grain elevators" (Liao et al. 2013).

Unfortunately, this synanthropic behavior can lead to adverse health effects. Similar to more common house dust mites such as *Dermatophagoides* species, *T. putrescentiae* is also known to be a human allergen. Sensitization to this species causes Immunoglobulin E (IgE) mediated reactions such as atopic dermatitis, rhinitis, and allergen induced asthma (Satitsuksanoa, 2016). Several different species-specific compounds such as proteins, frass, and proteases have been implicated as allergens through IgE tests, although only a few have been sequenced and confirmed (Erban et al., 2016). These components are classified in a few different allergen groups based on structure and cross reactivity (T. Perry, personal communication, January 13, 2023). Additional health concerns are the potential for pulmonary or interstitial acariasis (Qu et al., 2018)

Aspergillus flavus and *Tyrophagus putrescentiae* Interaction

Storage mites and fungi have a complex relationship within the stored grain ecosystem as both can thrive under approximately the same environmental conditions. *Tyrophagus putrescentiae* can live in temperatures ranging from 10-34°C. *Aspergillus flavus* overlaps with that range preferring temperatures in the 12-48°C. Both species thrive with humidity levels at or above 85%. Although somewhat tolerant of low humidity, maintaining high RH (Relative Humidity) is helpful for the growth and reproduction of *T. putrescentiae*. The mite has a poorly sclerotized cuticle, a large surface area to body ratio, and a water activity of >0.99 making them susceptible to desiccation (Eaton & Kells, 2009). Desiccation susceptibility is exacerbated in nymphal stages with die-offs seen in high temperatures despite high humidity levels (Sánchez-Ramos et al., 2007). Despite this, its broad temperature range and overall tolerance allow them to effectively disperse to different habitats without the need for a hypopal stage (Kheradmand et al., 2007).

Tyrophagus putrescentiae and *A. flavus* are both stored commodity pests contributing to degradation of stored grains through feeding, growth, and buildup of waste material (Malik et al, 2018). Together, mold and mite seem to have formed some sort of symbiotic relationship. Although both storage mites and fungi can live full lives in grain bins in the absence of each other, they are shown to have increased fecundity when in the presence of one another and rarely occur as a food hazard independently (Stejaskal et al., 2002).

In essence, *Aspergillus flavus* acts as a feeding facilitator and provides an excellent food source for the mites. Kernels of maize are impenetrable to *Tyrophagus* with an intact pericarp. Luckily, for the mites, the normal feeding process of *Aspergillus* breaks open seed coats allowing the mites access to the germ (Thomas and Dicke, 1971). As *Aspergillus* breaks down material, it predigests food, making an accessible nutrient dense meal for the mites (Bronswijk & Sinha,

1973). In addition to grain, the mites feed on fungal mycelium and spores. Fungal spores and hyphae contain phosphorus, nitrogen, vitamin b, and moisture sufficient to sustain populations (Franzolin et al., 1999; Qu et al., 2018). *Tyrophagus putrescentiae* has been documented as a more efficient fungivore than other grain infesting mites such as *Acarus siro* (Linnaeus,1758) in fact, a diet of *Aspergillus* alone has been confirmed to provide sufficient nutrition to sustain and increase *T. putrescentiae* populations (Sinha & mills, 1968; Van Asselt,1999; da Silva et al., 2019).

Despite ingestion, fungal spores can still be viable after excretion, providing a mechanical transport for the reproductive bodies throughout and between grain bins (Franzolin et al., 1999; Griffiths et al., 1959). The long hairs and dense populations of *T. putrescentiae* make the surface of the mites another way spores disperse. After deeper investigation, it was found that the composition of spores within the gut and the surface of *Tyrophagus* were essentially identical, indicating that rather than random adherence of spores to mite surfaces, the animals were actively seeking out specific fungi species (Hubert et al.,2003). *Tyrophagus putrescentiae* seem to gravitate towards stored grain fungi, many of which are medically important, like *A. flavus* (Hubert et al., 2003). Part of this interaction is that the fungi themselves actively draw in stored grain mites. Different volatiles have been identified as attractants to mite species (Van Asselt, 1999). Through volatile extract choice tests, storage mites seem to show preference for certain fungi, with *A. flavus* once again being one of the most preferred (Thomas & Dicke, 1971).

Purpose and Objectives

Given the economic and potential health ramifications that *A. flavus* and T. *putrescentiae* can cause when invading stored maize, further investigation into the interaction between these two organisms is needed. The complex network of known interactions between these two species sets

the stage for what could be an intricate dynamic affecting the population and growth of the organisms. The objectives of this thesis are as follows:

1. Assess the impact that growing in systems with *A. flavus* has on population growth and patterns of *T. putrescentiae* through counts of individuals.
2. Assess the impact that growing in systems with *T. putrescentiae* has on population growth and patterns of *A. flavus* through qPCR.
3. Determine if temperature affects growth patterns the organisms show while growing in the same system.

Materials and Methods

Maize Preparation

U.S. sample grade dent corn was obtained from the Atungulu lab at the University of Arkansas Department of Food Science and plated on potato dextrose agar to determine the species of mold contaminating the sample. The moisture content of the corn was determined by a laboratory oven over 48 hours with dried kernel weight subtracted from original weight. Before each trial, corn was sterilized by submerging in 95% ethanol (ETOH) and agitating for three minutes. Following sterilization, corn was left to dry overnight.

Tyrophagus putrescentiae Maintenance

Two *T. putrescentiae* colonies were obtained from the Philipps lab at Kansas State University and were maintained in quart jars sealed with filter paper and a rim. They were fed on a diet of dog food coated in a mite nutrition mixture. The mixture consisted of 475 mL water, 100 g Rachel Ray Nutrish dog food, 5 g Brewer's yeast, 5 g Alphacel, 3.5 – 4.5 g Agar (varying depending on thickness desired), 25 mL Glycerol, and 5 mL Methyl-p-benzoate (15% methyl-p-benzoate in 95% Ethanol) used as an antimicrobial and yeast growth limiting agent. To prepare, the ingredients were brought to a boil for ten minutes over medium heat adding the Methyl-p-benzoate solution ~two minutes before removal from the hot plate. The nutrition mixture was smeared along the sides of the jar and mixed into dry dog food to fully coat it.

Colonies were kept in a 10-gallon plastic bin filled halfway with water and placed in an incubator at 25°C with a relative humidity of 70-75%. Humidity was maintained by keeping additional trays of water within the incubator and monitored with a humidity gauge. More colonies were started every three weeks to four weeks as needed, with four kept at a time in the insect rearing facility building.

Aspergillus flavus Maintenance

Aspergillus flavus 21882 was obtained from the Agricultural Research Service Culture Collection (NRRL) and plated onto PDA. This particular strain was isolated from a peanut in Georgia, USA in 1991 by the National Peanut Research laboratory. After the first plates grew out, a glycerin stock was made to preserve the integrity of the fungus.

Experimental Trials

Sixty 16 oz mason jars were filled with 275 g of corn each and treated with four different treatment groups characterized as follows: A*spergillus* spores only, *Aspergillus* spores and *T. putrescentiae, T. putrescentiae* only, and plain corn. The treated jars were tested in two trials. In the first trial, the jars were kept at a temperature of 30°C. In the second, the jars were kept at 25°C. These represent optimum growth temperatures with *Aspergillus* growing well at 30 and *Tyrophagus* growing well at 25°C (Jayas & White, 2003). Filter paper and a mason jar rim was used to seal the jars in both. A saturated salt solution of Potassium Chloride was used to maintain RH around 84.34 ± 0.26 at 25°C and 83.62± 0.25 at 30° C (Greenspan, 1977).

To inoculate grain jars with a mite treatment, approximately 150 mites (~1 tsp) were added directly to the surface of the corn in the center. These amounts are adapted from the methods used in Franzolin et. al. (1999).

To prepare spore suspension, spores were harvested from plates into a falcon tube with a few mL of pure water using a flat lab spatula and a water wash. Spore numbers in the falcon tube were counted with a hemocytometer and water content was adjusted to get to a concentration of 3x10^7 spores/mL water (method adapted from Franzolin et al.,1999). Treatments containing

Aspergillus had 4 mL of spore suspension applied to the top center of the corn in each jar using a 5 mL pipette.

Sample Collection

In each trial, the jars were sampled at 4, 7, 14, 21, 35, and 42 days using a 1" diameter pipe to take a core from the center of the jar. Once a jar was sampled, it was scrapped to prevent further collection of data from a disturbed system. Trials were run from June 26, 2022 to August 04, 2022 and December 15, 2022 to January 25, 2023.

The samples were frozen and mixed, then divided in half with one portion going towards qPCR and the other for mite counts. Approximately 5 kernels from each sample dedicated to qPCR were shaken with three large metal beads for 3 minutes at 1500RPM in a 1600 miniG tissue homogenizer to obtain a fine powder for DNA extraction. Homogenized powder was stored in a -80°C freezer until ready for qPCR.

Absolute qPCR and Analysis

Genomic DNA was extracted from samples using a DNeasy plant pro kit by Omega Bio-tek. qPCR was run on samples using florescent dye-based detection (Mideros et. al 2009). qPCR samples containing just *Aspergillus* treatment were run using the primers Af2 forward primer: 5′-ATCATTACCGAGTGTAGGGTTCCT-3′; reverse primer: 5′-GCCGAAGCAACT AAGGTACAGTAAA-3′ A Power SYBR Green PCR Master Mix (Applied Biosystems) with template DNA and primers was run using an Analytik Jena qTower model qPCR machine with a PCR protocol adapted from Mideros et. al. (2009).

A standard curve was made for analysis of *A. flavus* DNA by running a serial 10-fold dilution of positive control DNA. The log of cycle threshold (Ct) values for each dilution were plotted against the log of the starting concentration to generate a line of best fit in Microsoft excel.

To verify assay efficiency, coefficient of determination (R^r) Can be calculated through the use of the standard curve and the following equations:

Five number summaries of the Ct values and multi factor ANOVAs were generated to visually compare treatment groups using JMP statistical software. Line graphs of Ct data were generated in Microsoft excel to visualize population trends over time.

Mite Counts and Analysis

Mite extraction methods were adapted from multiple methods mentioned in Monfreda et al. (2010). Samples were placed in an Erlenmeyer flask with enough water to fully submerge the material. Samples were left in the water for three minutes, gently swirling a few times each minute to dislodge any mites stuck to the kernels. Mites that floated to the surface of the water were poured off and rinsed in a fine mesh sieve. The rest of the sample was dumped into the top of layered sieves and rinsed to further dislodge any mites which were caught in the bottom fine mesh sieve. Mites collected in the sieves were then transferred to a petri dish using a wash bottle. The process was repeated three times for each sample to ensure all mites were extracted (Monfreda et al. 2010).

Extracted mites were suspended in water in a petri dish and photographed on a black background to ensure visibility. Photos were uploaded to DotDotGoose software (https://www.amnh.org/research/center-for-biodiversity-conservation/research-and-conservation/biodiversity-informatics/software-counting-images-open-source) where individual mite counts were performed. The mite counts of individuals per sample were used in R studios to generate locally weighted scatter plot smoothing curves (LOESS), a combination of nonlinear regression and least squares analysis to provide a visualization of population trends in each

treatment group over time. JMP statistical software was used to perform multifactor ANOVAs between treatments, temperature, and time (days) and fit Spline curves to the data.

Results

Following two weeks of observation, the subset of plated unsanitized corn contained the following species: *Trichoderma* spp., *Penicillium* spp., *Phomopsis* sp., *Fusarium* spp., and *Aspergillus niger*. The moisture content of the corn was determined to be 18.903%

Mite Populations

The number of individuals in samples during trials run at 25°C was significantly higher than their equivalent day samples held at 30°C (Table 1). This held true for both of the mite containing treatment groups at this temperature. An ANOVA comparing number of individuals in samples between temperature treatments (Figure 1) shows a p>F of <.0001 leading to a rejection of the null hypothesis that temperature does not affect *T. putrescentiae* population growth.

The overall numbers of individual mites did not show a statistical difference between samples containing treatments with just *Tyrophagus* and samples treated with both *Tyrophagus* and *Aspergillus*. However, their population growth did show different patterns depending on treatment. Samples treated with just *Tyrophagus* displayed a linear growth over time. This was seen at trials run at both 25° C and 30 ° C. Mite populations increased faster in trials run at 25° C with their linear line of best fit displaying a slope of 131.46. This contrasts with a slope of 57.591 seen in the 30 ° C trials. (Figure 2)

Treatments containing both mites and mold displayed a population boom followed by an abrupt drop in number. This was again seen in both the 25°C and 30°C trials, but their population decline hit at different sample days depending on the temperature. The population peaks

followed by decline occurred at 14 days in the 25°C trial and at 21 days in the 30°C trial (Figure 3).

These population growth trends led to significant differences between treatments on certain days. A significant p value: 0.0209 was produced from an ANOVA for day 14 in the 25°C trial where the major population spike and subsequent decline was seen (Figure 4). In the 30°C trial, no significance was seen during the population spike at 21 days (Figure 5). Both temperature trials resulted in significant p values on the ending sample day 42 (Figure 6- 7).

Aspergillus Ct Values

Overall, the Ct showed a decreasing trend as time went on in all sample groups, indicating an increase in *Aspergillus* growth. Both treatments grown at 25°C showed a larger spread in Ct values with ranges of 0.312 with *Aspergillus* on its own and 0.285 in the *Aspergillus* and *Tyrophagus* treatment (Figure 8). Through the use of an ANOVA, it was found that temperature was statistically significant with lower Ct values found at 30°C indicating more growth (Figure 9). When Ct values were plotted, treatments grown at 30°C invariably showed lower numbers up until the 35-day mark at which values had a decrease in decline and were outpaced by treatments grown at 25°C (Figure 10). At the 42-day mark, both temperature trials showed a significant difference between treatment groups with a p-value of 0.0132 at 25°C (Figure 11) and p= 0.0179 at 30°C (Figure 12). Although different growth was observed at different temperatures, overall, there was no significance between treatment groups.

Discussion

The temperature was an important variable affecting mite population growth in this study. Mite populations did not grow as much at 30°C in both treatment groups. This makes sense given the mites tendency to desiccate at higher temperatures. Egg and larval mite stages have the highest mortality rate at hot temperatures, stages whose absence would make population growth slowed as seen reflected in the data (Sánchez-Ramos et al. 2007)

Based on the data it can be thought that there is a kindof symbiosis going on between *T. putrescentiae* and *A. flavus*. When the organisms are together in treatment groups, there is an obvious change in population growth seen when looking at graphs of counted individual mites. The Ct values gathered from the *Aspergillus* with *Tyrophagus* treatments appear to follow the general growth or decline of mite populations seen in their respective samples up until the 21-day mark. Notably, the major population peaks and declines seen in the mite populations are reflected in the Ct data as well. As previously stated, sample days where the mite population shows interesting growth, such as days when the population peaks (21 and 14) and sample day 42, show significance between mite numbers in different treatment groups. This is not seen in the 30°C trial at the population peak at 21 days or on the final day 42. On those two particular sample days, one microcosm core from each had a reasonably higher or lower number of mites than the other two, creating an outlier that likely led to the insignificance between treatment groups (Figure 5, Figure 7). Overall, the mite population has shown a decline in growth after hitting peak population at 21 days. The mirroring growth trends seen shed light on the complex symbiosis between the two organisms. With many factors at play, it is hard to determine the true relationship at hand, but a few possible explanations are explored below.

It is worth noting that mites collected from samples at and after the 21-day mark had *A. flavus* growing in them, indicating the mold had started to break down the bodies of mites. Since samples were frozen before mite extraction, there was no way to know if the mold was colonizing the mites while they were still living or, if it was just decomposing already deceased animals. *Aspergillus flavus* and a few other species of A*spergillus* are known to be entomopathogenic, effecting a wide variety of creatures such as aphids, mosquitos, silkworms, as well as being one of the causal agents for stonebrood (Abrar, 2020; Jaber et al. 2016; Wang et al. 2019; Bava et al. 2022). The entomopathogenic qualities of *Aspergillus* have not been extensively explored in respect to mites, but one study found that the closely related *A. oryzae* effectively colonized and killed the Red Poultry mite (*Dermanyssus gallinae* (De Geer, 1778) (Wang et al. 2019). Despite numerous studies conducted on these two organisms' interactions, to the best of my knowledge, there has been no report of *A. flavus* colonizing and killing living *T. putrescentiae.*

Tyrophagus putrescentiae has been documented as having an intricate relationship with a few species, including many molds and a protist. Michalczyk-Wetula et al. (2021) describe *T. putrescentiae's* interaction with various slime molds as "an interesting system of mutual interactions bearing symptoms of several known ecological interactions (i.e., parasitism, pseudo-parasitism, parasitoidism, insidious predation, grazing, trophic competition, necrophagy, mutualism, and phoresy) at various stages of their life cycle.". While the slime molds in this paper did not seem to have much of an interaction with *Tyrophagus* beyond serving as a food source, the interrelationship the mite shares with *Aspergillus* seems to be much more complicated. Vogel et al. (2021) reports on *T. putrescentiae* increasing mycotoxin production from various mold species in multiple kinds of stored grains. They hypothesize that *Tyrophagus*

bringing in many kinds of molds may induce species to produce mycotoxins in competition. While the exact involvement of mycotoxin in this interaction is not known, at minimum the increase in these toxins shows molds (*Aspergillus* included) growing alongside *T. putrescentiae* behave differently than when without acarid roommates.

Another interaction to consider in this growth pattern is active transport. Active transport by *Tyrophagus* has been documented with many species of molds and other small organisms, but particularly with storage molds of medical importance (Franzolin et al. 1999; Griffiths et al. 1959; Vogel et al. 021). The mite is able to transport spores while in the alimentary canal and on the surface of the exoskeleton and setae (Hagstrum, 2016). An interesting active transport case between *Tyrophagus* sp. and two protists, *Tetmemena* sp. (Eigner, 1999) and *Sterkiella* sp. *(*Foissner et al. 1991) was recently described (Bharti et al. 2020). Protist ciliates had been seen attaching to the dorsal surface of *Tyrophagus* while detritus feeding in wet conditions and being transported to dryer areas to complete their life cycle. In turn, the mites feed on the overabundance of ciliates mixed in with the detritus (Bharti et al. 2020). The variety of microorganisms *Tyrophagus* can transport shows their competence as vectors in multiple environments. When looking at the increase and decline of *Aspergillus* population, active transport of fungal spores in the initials to aid the growth of mold would depend on the number of mites in the system. In that case, it would make sense that until mold is fully established in a system, its growth patterns would follow that of one of its mechanisms of spread.

Coinciding populations could also be explained by stimulated fungal growth in response to *Tyrophagus* feeding. Similar to herbivore-induced changes in plants, some fungi have shown a switch in polymorphism and stimulated growth rates in response to collembolan feeding (Hedlund et al. 1991; Bengtsson et al. 1993). Fragmentation and size of fragments, as well as

normal growth rates, influence compensatory growth. The collembola as a group interact with molds in the soil similarly to mites, acting as an active transport system for spores. Compensatory herbivore induced growth does not produce spores but allows fungi to potentially survive damage. Compensatory growth of *Aspergillus* in response to grazing has not been investigated but with a similar mold and arthropod system, there could be something similar at play.

The mite's diverse array of reported interactions indicates the species is one inclined to form complex relationships with a variety of microorganisms. The combination of active transport of spores along with the possibility of compensatory growth would explain the correlating growth patterns of the two species up until the 21-day mark.

The decline of mite numbers seen in samples after reaching their population peaks may have been influenced by multiple factors. The possibility that they had reached carrying capacity for the number of resources in their small microcosms would explain their population decline. Studies of rearing *Tyrophagus* under laboratory conditions showed that populations increased sharply until the 8-week mark (Ammar et al. 2021). Comparatively, the mite population decline seen in samples with *Aspergillus* at 14 and 21 days is very fast. Treatments where T. *putrescentiae* were allowed to grow on their own showed no sign of a population drop at the 42-day mark, indicating that the presence of A*spergillus* in the system caused the population drop to occur sooner. Many mites dying off after such a large feeding pressure would explain why the Ct values of *A. flavus* increased and stopped following the general population trends of the mites after the 21-day mark. At this point, *A. flavus* had sufficient transport from *Tyrophagus* and time to establish their population and started to outcompete or purposely kill off the mites.

Mycelial defense mechanisms have been identified in many species such as the basidiomycete *P. ostreatus* (Jacq. ex Fr.) P.Kumm. (1871). The mushroom has been documented mounting a response to broad injury involving the MAMP and DAMP pathways. Feeding by *T. putrescentiae* specifically induced the MAPK signaling pathways, altered JA and NEJA contents, and induced gene expression of terpenoid and steroid biosynthesis (Li et al. 2022). These synthesized compounds repelled *T. putrescentiae*, protecting the fungus from being fed on. Although unrelated to *A. flavus*, it is possible that there are some similar defense mechanisms employed in response to fungivore feeding as a result of longtime interaction with mites. These prolonged antagonisms or even mutualism steer coevolution between species over time (Künzler, 2015). Given that *A. flavus* can reasonably be assumed to naturally encounter *T. putrescentiae* in more than a few of its known normal environments and sustain feeding damage, a chemical defense of some sort is not out of the question.

Currently, the aspergilli have a few known chemical defenses. Gliotoxin is produced to target other fungi, while aflatoxin B1 and α-Sarcin are toxins secreted against arthropods (Drott et al. 2017; Künzler, 2018). *Aspergillus nidulans* (G Winter, 1884) was found to secrete toxins in the fruiting bodies commonly targeted by arthropods (Stotefeld et al. 2012). While in the case of this study where the participating strain of *Aspergillus flavus* is not an aflatoxin producer, it does not rule out the possibility of other chemical defenses that may have been acting against the mites. In fact, a field sampling survey of the USA found that 29% of all isolates were nonaflatoxogenic, a substantial number to be left without a substituting anti-arthropod defense (Horn & Dorner, 1999). Possible chemical defenses given off in response to a large population growth and the pressure from herbivory that comes with that could be a reason for the rapid population decrease seen in the samples. On the other hand, adapted immunity to chemical defense in fungivores is

something to be considered. Although unexplored in *Tyrophagus*, various species of Collembola have been documented altering feeding behavior based on hyphal toxin concentration and therefore avoiding ingesting lethal levels. They have also been seen to detoxify sterigmatocystin injested during feeding (Staaden et al. 2010; Stotefeld et al. 2012). Both these behaviors were documented with soil fungi found in their environment.

Competition for food resources would also be a factor contributing to the sudden decline in mite populations and continued growth of mold. With the fungi growing steadily along with the mite population, by the 21-day mark there would have been a large number of organisms vying for food in a closed system. Overgrowth of *Aspergillus* in the mason jar combined with corn fully inundated with mold and now rotting, is no longer as attractive as a food source to *Tyrophagus.* Although often reported to feed on decaying matter, my observations through raising *T. putrescentiae* for ~two years would add a caveat to that statement. *Tyrophagus* seems to only feed on decaying matter in the early to mid-stages of decomposition. After heavy decomposition fluids start to be excreted from the rotting matter and the mite no longer seems to feed.

Conclusion

Tyrophagus putrescentiae and *A. flavus* have a complex relationship within the stored grain ecosystem that has yet to be fully explored. My experimentation attempted to delve deeper into this symbiosis and begin to understand the interactions.

The difference seen in growth patterns of the two organisms grown in the same treatment group versus those grown individually shows that the two organisms affect how each other grow over time. *Tyrophagus* and *Aspergillus* showed mirroring growth and decline of population until the 21-day mark when the mites experienced a spike in population growth. After this date, *A. flavus* continued to grow at a slow steady rate while the mite's population declined.

This relation could be due to a variety of different factors, but it can be hypothesized that a combination of any number of components discussed above could lead to such population growth. The initial growth of both populations represents a time where the mites were experiencing little pressure from overcrowding and had an abundance of food from both available maize and hyphae. During this time, the mites traveling around within the system likely helped the *Aspergillus* spread throughout the jars at a faster rate. It is possible that hyphal feeding from the mites could have also contributed to increased growth by stimulating the mold metabolism, but this has not been investigated with the organisms involved in this experimentation.

The mite populations peak and then subsequently decline likely due to overcrowding and lack of resources. Another possibility is that the *Aspergillus* was producing some kind of anti-herbivory defensive compound that reached levels high enough to impact the mite populations. The molds

continued growth shows an established population that no longer needs whatever benefits the mites bring them.

Regardless of the mechanisms at play, the presence of *Aspergillus flavus* in the system affected the population growth of the mites causing a rapid increase and an early decline in populations. Although the *Aspergillus* Ct values showed the mold population following the mite population trends early on, overall, there was no major difference in growth between treatments involving both organisms and those with mold only.

The results add to what is known about the symbiotic relationship between *Aspergillus flavus* and *Tyrophagus putrescentiae* within the stored grain ecosystem. Further investigation into this system is needed to determine the exact mechanisms causing this interaction.

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Table 1. Tyrophagus putrescentiae numbers

Sample Day	Sample #	Treatment	Mite#	Temperature
4	1	TA	1008	25
4	2	TA	1481	25
4	3	TA	1492	25
7	1	TA	1503	25
7	2	TA	1208	25
7	3	TA	1113	25
14	1	TA	6959	25
14	2	TA	5286	25
14	3	TA	5657	25
21	1	TA	2924	25
21	2	TA	4596	25
21	3	TA	5374	25
35	1	TA	3637	25
35	2	TA	4737	25
35	3	TA	3948	25
42	1	TA	4142	25
42	2	TA	3805	25
42	3	TA	3933	25
4	1	T	1174	25
4	2	T	986	25
4	3	T	756	25
7	1	T	1332	25
7	2	T	902	25
7	3	T	1308	25
14	1	T	2946	25
14	2	T	3418	25

Table 1. continued Tyrophagus putrescentiae numbers

Sample Day	Sample #	Treatment	Mite#	Temperature
14	3	T	4331	25
21	1	T	3178	25
21	2	T	4711	25
21	3	T	4749	25
35	1	T	4895	25
35	2	T	5802	25
35	3	T	4829	25
42	1	T	6722	25
42	2	T	5217	25
42	3	T	6444	25
4	1	TA	1249	30
4	2	TA	1167	30
4	3	TA	917	30
7	1	TA	1259	30
7	2	TA	1376	30
7	3	TA	1472	30
14	1	TA	980	30
14	2	TA	1428	30
14	3	TA	1318	30
21	1	TA	2647	30
21	2	TA	2550	30
21	3	TA	2501	30
35	1	TA	473	30
35	2	TA	522	30
35	3	TA	567	30
42	1	TA	535	30

Table 1. continued. Tyrophagus putrescentiae numbers

Sample Day	Sample #	Treatment	Mite#	Temperature
42	2	TA	332	30
42	3	TA	375	30
4	1	T	91	30
4	2	T	360	30
4	3	T	447	30
7	1	T	1260	30
7	2	T	836	30
7	3	T	1735	30
14	1	T	1405	30
14	2	T	2135	30
14	3	T	1311	30
21	1	T	753	30
21	2	T	978	30
21	3	T	2298	30
35	1	T	1849	30
35	2	T	2812	30
35	3	T	2008	30
42	1	T	1815	30
42	2	T	4310	30
42	3	T	2615	30

Table 2. *Aspergillus flavus* Ct values

Sample Day	Sample #	Treatment	Ct	log Ct	Temperature
4	1	TA	21.47	1.33183	25
4	2	TA	19.23	1.28397	25
4	3	TA	20.68	1.31555	25
7	1	TA	22.04	1.34321	25
7	2	TA	19.65	1.29336	25
7	3	TA	20.8	1.31806	25
14	1	TA	15.98	1.20357	25
14	2	TA	14.85	1.17172	25
14	3	TA	15.87	1.20057	25
21	1	TA	11.42	1.05766	25
21	2	TA	17.99	1.25503	25
21	3	TA	18.86	1.27554	25
35	1	TA	13.59	1.13321	25
35	2	TA	17.09	1.23274	25
35	3	TA	18.86	1.14082	25
42	1	TA	13.47	1.12936	25
42	2	TA	12.76	1.10585	25
42	3	TA	12.82	1.10788	25
4	1	A	20.04	1.30189	25
4	2	A	20.51	1.31196	25
4	3	A	19.78	1.29622	25
7	1	A	16.72	1.22323	25
7	2	A	21	1.32221	25
7	3	A	20.79	1.31785	25
14	1	A	16.52	1.21801	25
14	2	A	17.61	1.24575	25

Table 2 continued. *Aspergillus flavus* Ct values

Sample Day	Sample #	Treatment	Ct	log Ct	Temperature
14	3	A	19.18	1.28284	25
21	1	A	20.5	1.31175	25
21	2	A	14.44	1.15956	25
21	3	A	14.58	1.16375	25
35	1	A	13.31	1.12417	25
35	2	A	17.57	1.24477	25
35	3	A	10.51	1.02160	25
42	1	A	11.67	1.06707	25
42	2	A	10.86	1.03582	25
42	3	A	10.24	1.01029	25
4	1	TA	17.06	1.23198	30
4	2	TA	20.01	1.30125	30
4	3	TA	17.26	1.23704	30
7	1	TA	13.9	1.14301	30
7	2	TA	14.47	1.16047	30
7	3	TA	13.15	1.11893	30
14	1	TA	14.6	1.16435	30
14	2	TA	13.16	1.11926	30
14	3	TA	14.23	1.1532	30
21	1	TA	13.46	1.12905	30
21	2	TA	13.14	1.1186	30
21	3	TA	13.82	1.14051	30
35	1	TA	12.34	1.09132	30
35	2	TA	12.56	1.09899	30
35	3	TA	11.8	1.07188	30
42	1	TA	11.59	1.06408	30

Table 2. *Continued*. *Aspergillus flavus* Ct values

Sample Day	Sample #	Treatment	Ct	log Ct	Temperature
42	2	TA	11.97	1.07809	30
42	3	TA	12.36	1.09202	30
4	1	A	16.27	1.21139	30
4	2	A	15.64	1.19424	30
4	3	A	17.4	1.24055	30
7	1	A	16.5	1.21748	30
7	2	A	16.49	1.21722	30
7	3	A	13.77	1.13893	30
14	1	A	15.55	1.19173	30
14	2	A	14.76	1.16909	30
14	3	A	13.77	1.13893	30
21	1	A	12.93	1.1116	30
21	2	A	15.08	1.1784	30
21	3	A	12.97	1.11294	30
35	1	A	13.85	1.14145	30
35	2	A	14.43	1.15927	30
35	3	A	13.63	1.1345	30
42	1	A	13.33	1.12385	30
42	2	A	12.84	1.10857	30
42	3	A	13.74	1.13799	30
4	C	C	33.25	1.52179	30
7	C	C	34.59	1.53895	30
14	C	C	33.88	1.52994	30
21	C	C	No Ct	No Ct	30
35	C	C	No Ct	No Ct	30
42	C	C	33.8	1.52892	30

Table 3. increase or decrease in values per sample day at 25° C

	Mold Population	Mite Population
4	-	-
7	up	up
14	down	down
21	up	up
35	up	down
42	Slight increase	down

Table 4. increase or decrease in values per sample day at 30° C

Day	Mold Population	Mite Population
4	-	-
7	up	up
14	up	up
21	down	down
35	Slight increase	down
42	Slight increase	down

Figure 1. ANOVA of Individual mite number by temperature treatment

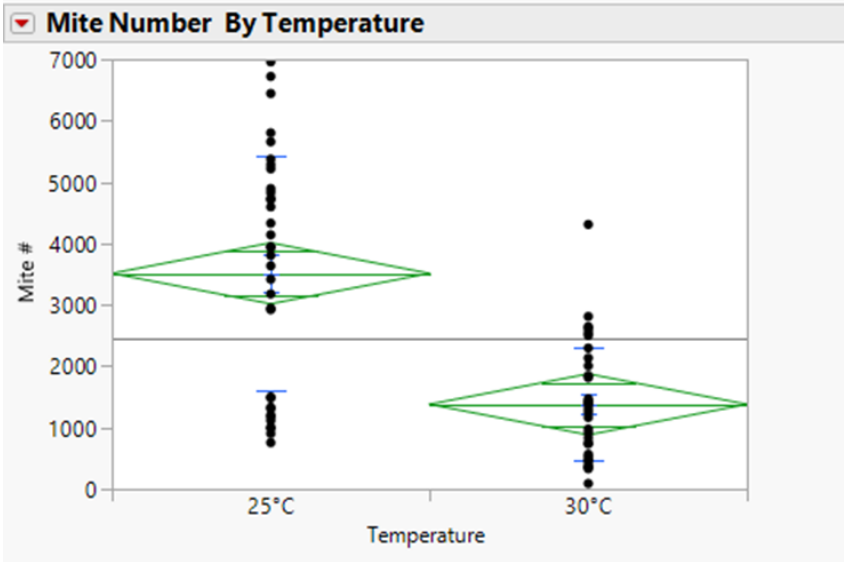

Figure 2. Number of individual *Tyrophagus* in mite only treatment over time.

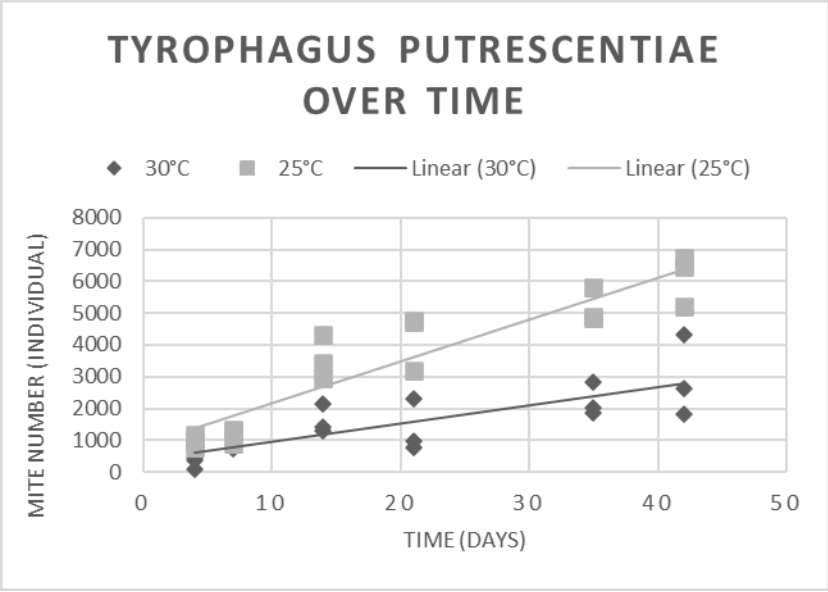

Figure 3. Individual numbers of *Tyrophagus* in the Mites and Mold Treatment. Error bars represent standard error.

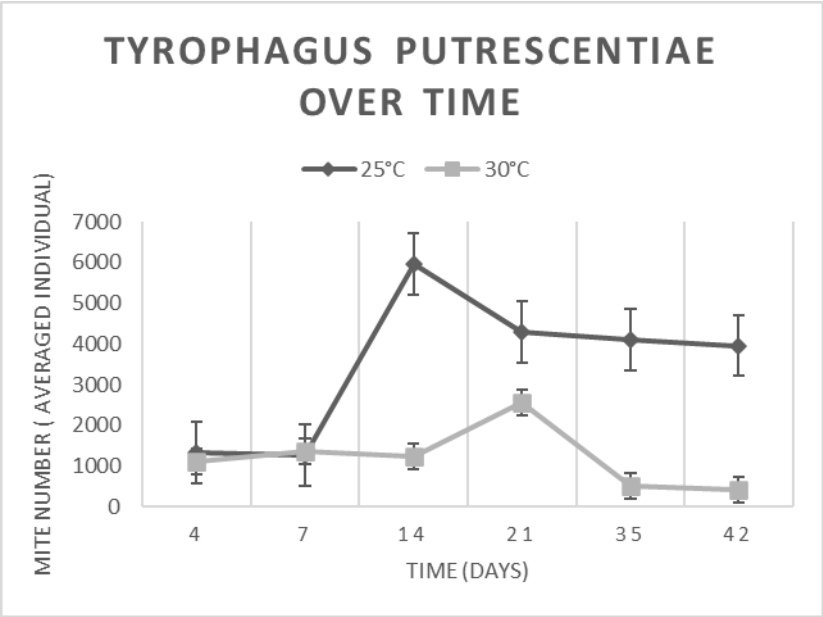

Figure 4. ANOVA of Individual *Tyrophagus* number by treatment at 14 days during the 25° C trial

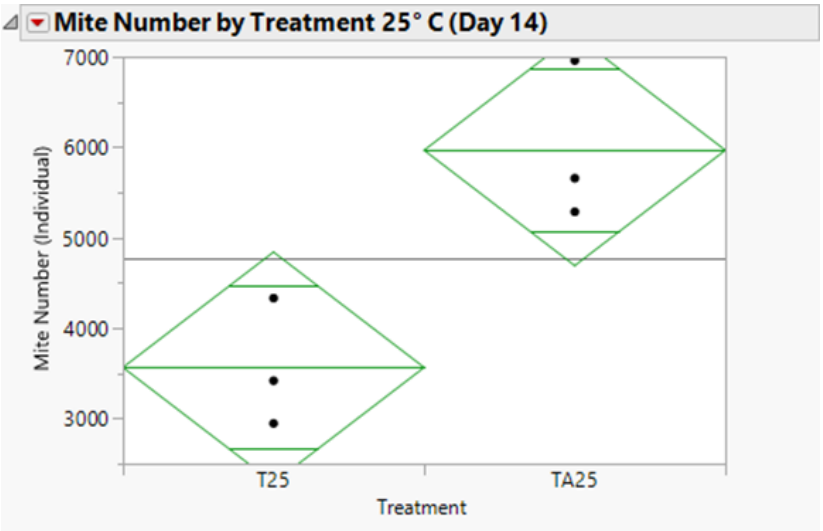

Figure 5. ANOVA of Individual *Tyrophagus* number by treatment at 21 days during the 30° C trail

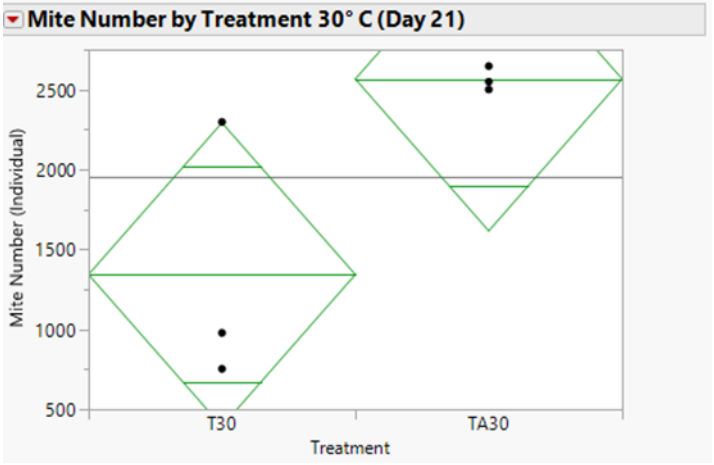

Figure 6. ANOVA of Individual *Tyrophagus* number by treatment at 42 days during the 25° C trail

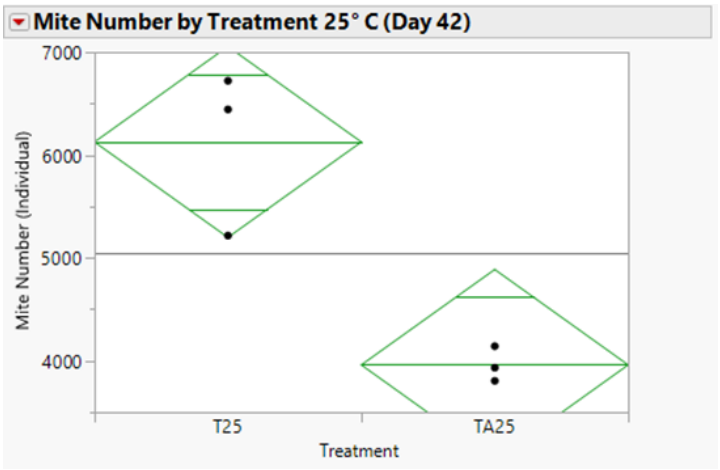

Figure 7. ANOVA of Individual *Tyrophagus* number by treatment at 42 days during the 30° C trail

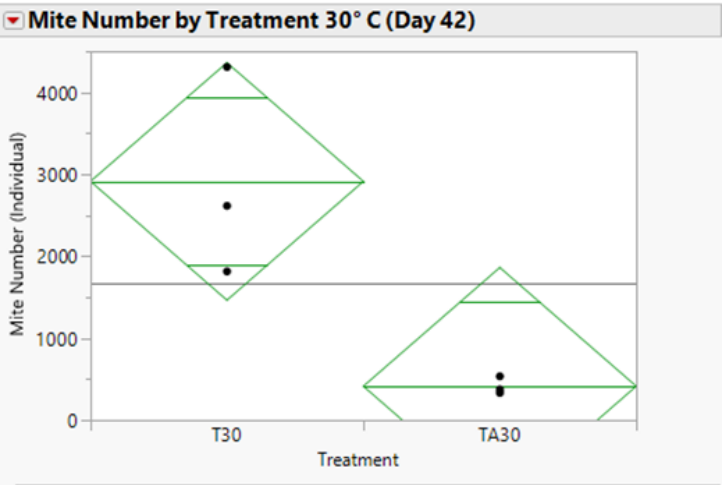

Figure 8. Boxplots comparing *Aspergillus* log Ct between treatment groups.

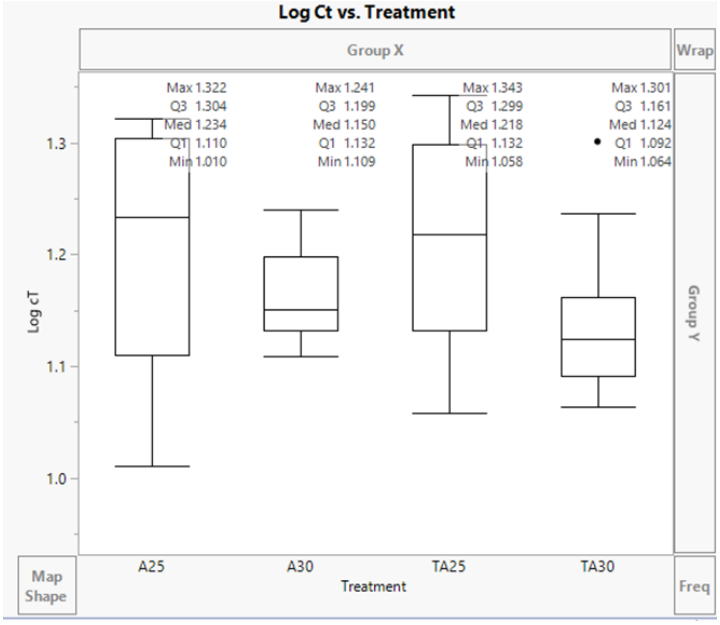

Figure 9. ANOVA of *Aspergillus* log Ct values by Treatment

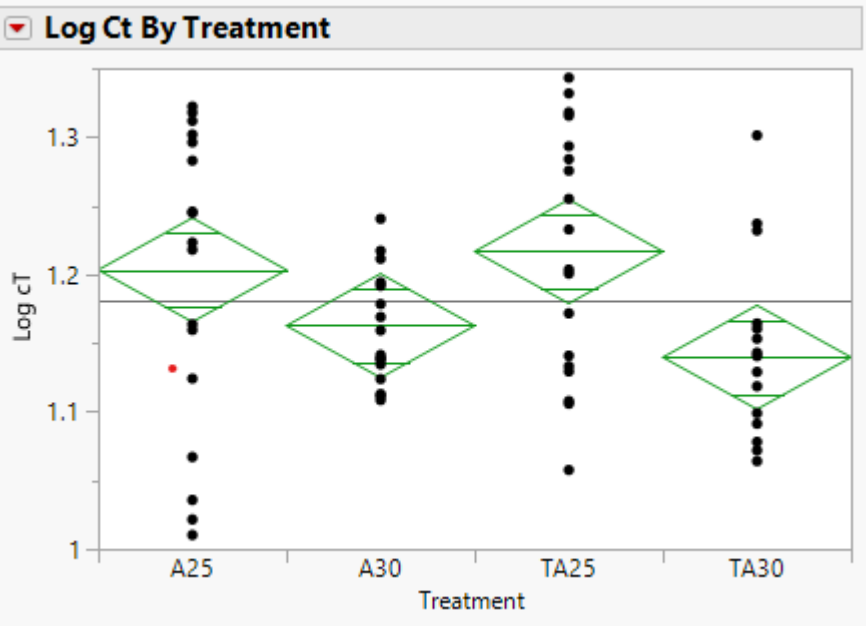

Figure 10. *Aspergillus* log Ct over Time. Error bars represent standard error.

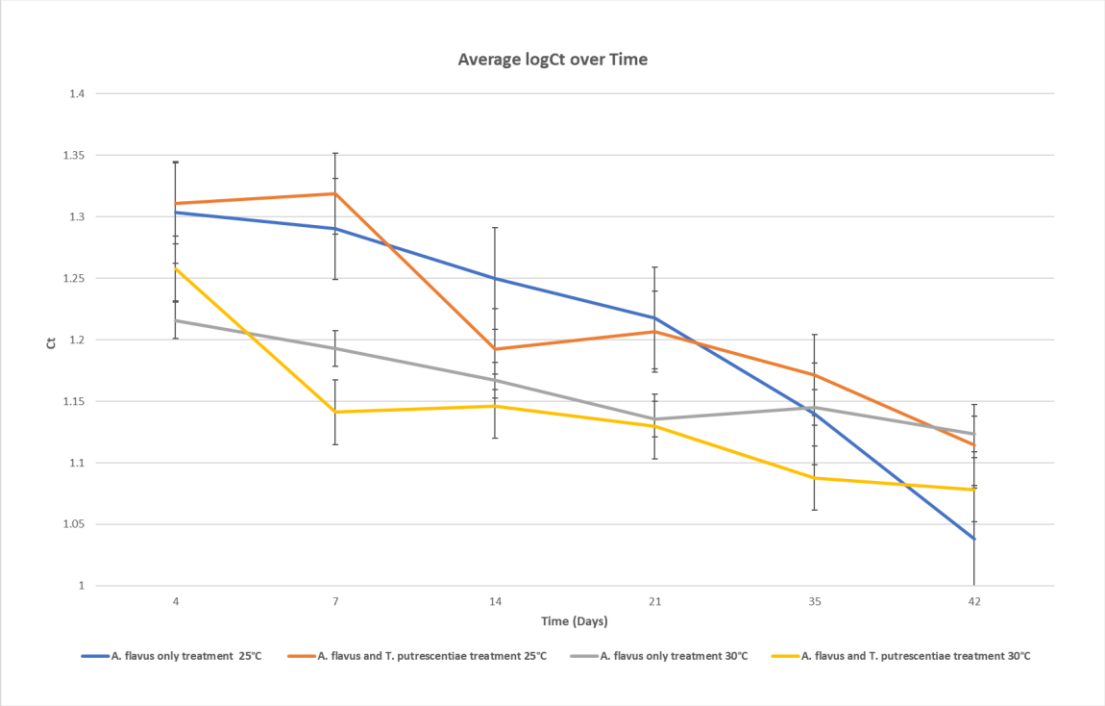

Figure 11. ANOVA of *Aspergillus* log Ct values by Treatment at 42 days during the 25° C

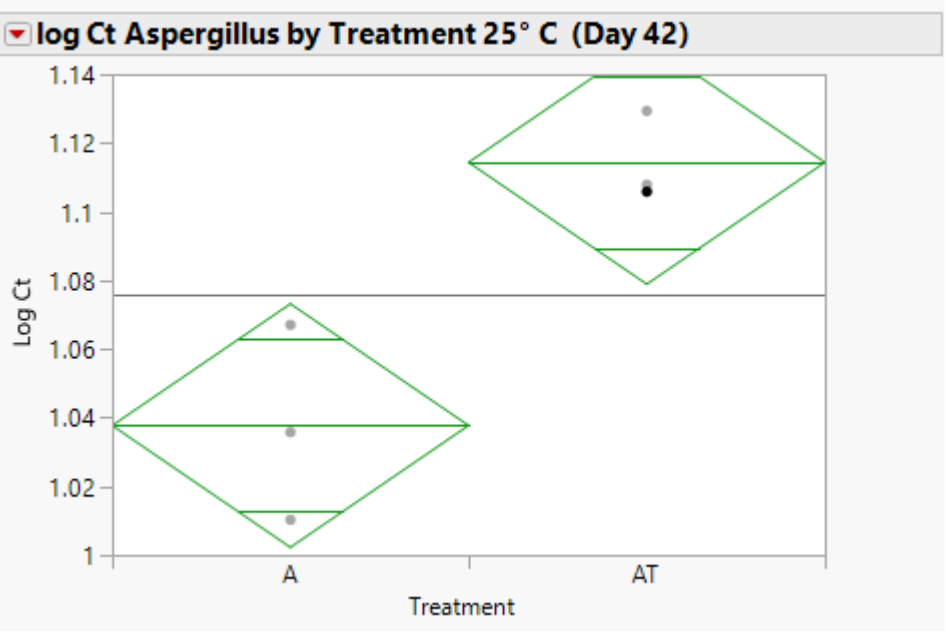

Figure 12. ANOVA of *Aspergillus* log Ct values by Treatment at 42 days during the 30° C