NTP

National Toxicology Program

U.S. Department of Health and Human Services

NTP Technical Report on the Toxicity Study of

Stachybotrys chartarum (CASRN 67892-26-6) Administered by Inhalation to B6C3F1/N Mice

NTP TOX 107

OCTOBER 2024

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Toxicity Report 107

October 2024

Foreword

The National Toxicology Program (NTP), established in 1978, is an interagency program within the Public Health Service of the U.S. Department of Health and Human Services. Its activities are executed through a partnership of the National Institute for Occupational Safety and Health (NIOSH, part of the Centers for Disease Control and Prevention), the Food and Drug Administration (FDA, primarily at the National Center for Toxicological Research), and the National Institute of Environmental Health Sciences (NIEHS, part of the National Institutes of Health), where the program is administratively located. NTP offers a unique venue for the testing, research, and analysis of agents of concern to identify toxic and biological effects, provide information that strengthens the science base, and inform decisions by health regulatory and research agencies to safeguard public health. NTP also works to develop and apply new and improved methods and approaches that advance toxicology and better assess health effects from environmental exposures.

The Toxicity Report series began in 1991. The studies described in the NTP Toxicity Report series are designed and conducted to characterize and evaluate the toxicological potential of selected substances in laboratory animals (usually two species, rats and mice). Substances (e.g., chemicals, physical agents, and mixtures) selected for NTP toxicity studies are chosen primarily on the basis of human exposure, level of commercial production, and chemical structure. The interpretive conclusions presented in the Toxicity Reports are derived solely from the results of these NTP studies and should not be misconstrued to represent an official policy of the individual agencies that participate in the NTP partnership (NIEHS, NIOSH, or FDA). Extrapolation of these results to other species, including characterization of hazards and risks to humans, requires analyses beyond the intent of these reports. Selection for study per se is not an indicator of a substance's toxic potential.

NTP conducts its studies in compliance with its laboratory health and safety guidelines and Food and Drug Administration Good Laboratory Practice Regulations and meets or exceeds all applicable federal, state, and local health and safety regulations. Animal care and use are in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. Studies are subjected to retrospective quality assurance audits before they are presented for public review. Draft reports undergo external peer review before they are finalized and published.

NTP Toxicity Reports are available free of charge on the NTP website and cataloged in PubMed, a free resource developed and maintained by the National Library of Medicine (part of the National Institutes of Health). Data for these studies are included in NTP's Chemical Effects in Biological Systems database.

For questions about the reports and studies, please email NTP or call 984-287-3211.

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Peer Review

The National Toxicology Program (NTP) conducted a peer review of the draft *NTP Technical Report on the Toxicity Study of Stachybotrys chartarum (CASRN 67892-26-6) Administered by Inhalation to B6C3F1/N Mice* by letter in January 2024 by the experts listed below. Reviewer selection and document review followed established NTP practices. The reviewers were charged to:

1. Peer review the draft *NTP Technical Report on the Toxicity Study of Stachybotrys chartarum (CASRN 67892-26-6) Administered by Inhalation to B6C3F1/N Mice.*
2. Comment on NTP's interpretations of the data.

NTP carefully considered reviewer comments in finalizing this report.

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Publication Details

Acknowledgments

Abstract

Stachybotrys chartarum, also known as "black mold," is a cellulolytic saprophyte with a worldwide distribution. Public concern for potential illnesses associated with water-damaged indoor environments has been heightened since the report of pediatric acute idiopathic pulmonary hemorrhage/hemosiderosis cases in the United States and following recent natural disasters. Although mycotoxicosis and pulmonary immunological endpoints have been previously examined, the systemic toxicity following subchronic inhalation of viable *S. chartarum* remains undercharacterized. To evaluate the toxicological responses to *S. chartarum*, B6C3F1/N mice were exposed to viable *S. chartarum* conidia (spores) twice a week for 3 months. All in-life procedures, including inhalation exposure, test article preparation, and hematology analysis, were completed by the National Institute for Occupational Safety and Health (NIOSH, Morgantown, WV). Battelle (Columbus, OH) conducted terminal necropsies, measured terminal body and organ weights, and evaluated gross lesions onsite at the NIOSH facility. Tissue processing and histopathology were completed at Battelle. Genetic toxicology studies on mouse peripheral blood erythrocytes were conducted by Integrated Laboratory Systems, LLC (Research Triangle Park, NC).

During the 3-month exposure study, groups of 10 male and 10 female B6C3F1/N mice repeatedly inhaled an estimated pulmonary dose of 1×10^4 viable *S. chartarum* conidia, 1×10^4 nonviable conidia (heat-inactivated particle control), or high-efficiency particulate air [filter] (HEPA)-filtered air. With the exception of one male mouse in the air control group, all mice survived to study termination. There was no effect of exposure on body weights. No gross lesions were observed at study termination. Mean absolute and relative lung weights were higher in male mice (11% and 5%, respectively) and significantly increased in female mice (33% and 35%, respectively) exposed to viable *S. chartarum* compared to the air control group.

Nonneoplastic lesions were observed in the larynx, lung, and bronchial lymph nodes. In males and females exposed to viable *S. chartarum*, nonneoplastic lesions were restricted mainly to the larynx and lung; however, there was a small incidence of lymphocyte hyperplasia in the bronchial lymph nodes of *S. chartarum*-exposed female mice (25%). In the larynx, epithelial squamous metaplasia was present at the base of the epiglottis in both males and females exposed to viable *S. chartarum*, whereas exposure to heat-inactivated particle control conidia did not significantly affect the larynx. The increased lung weights in the viable *S. chartarum*-exposed groups corresponded histopathologically with perivascular chronic-active inflammation, histiocytic cellular infiltration, medial hypertrophy of small- to medium-sized pulmonary arteries, goblet cell metaplasia, and bronchiolar epithelium hyperplasia in all exposed males and females. Lymphocyte hyperplasia of the bronchus-associated lymphoid tissue (BALT) was also present in the majority of viable *S. chartarum*-exposed males and females. Thrombi within pulmonary arteries were present in a limited number of *S. chartarum*-exposed males and females. The pulmonary histopathology of mice in the heat-inactivated particle control groups exhibited no lesions. Grocott's methenamine silver (GMS)-stained lung sections of viable *S. chartarum*-exposed mice revealed conidia within the alveoli and alveolar macrophages. In the bronchial lymph nodes, lymphocyte hyperplasia and conidia were additionally observed in two viable *S. chartarum*-exposed female mice.

Under the conditions of this 3-month study, target organs identified in B6C3F1/N mice following inhalation exposure to viable *S. chartarum* conidia were the larynx, lung, and (to a lesser extent)

the bronchial lymph nodes. The histopathological changes observed following viable *S. chartarum* exposure were not seen in the heat-inactivated particle control or air control mice. These results build on initial pulmonary immunology studies conducted at NIOSH using the same exposure parameters and demonstrate that the immunological responses and histopathology could be mediated by the viable *S. chartarum* bioaerosol.

Synonyms: *Stachybotrys chartarum* (*S. chartarum*); *Stachybotrys atra* (*S. atra*); *Stachybotrys alternans* (*S. alternans*); *Stachybotrys chartarum* (Ehrenb.) S. Hughes 1958; anamorphic, satratoxin-producing chemotype (CBS 414.95; IBT 9460)

Summary of Findings Considered Toxicologically Relevant in Male and Female Mice Exposed to *Stachybotrys chartarum* by Inhalation for Three Months

	Male Heat-inactivated Particle Control	Male Viable *S. chartarum*	Female Heat-inactivated Particle Control	Female Viable *S. chartarum*
Exposure Concentration (Estimated Lung Deposition)	1×10^4 heat-inactivated *S. chartarum* conidia	1×10^4 viable *S. chartarum* conidia	1×10^4 heat-inactivated *S. chartarum* conidia	1×10^4 viable *S. chartarum* conidia
Survival Rates	10/10	10/10	10/10	10/10
Body Weights	No effect	No effect	No effect	No effect
Clinical Findings	None[a]	None	None	None
Organ Weights	None	None	None	↑ Absolute and relative lung weights compared to either air control or heat-inactivated particle control
Hematology	None	↑ Eosinophils compared to either air control or heat-inactivated particle control	None	None
Nonneoplastic Effects	None	<u>Larynx:</u> epiglottis, metaplasia, squamous (4/10) <u>Lung:</u> perivascular, inflammation, chronic-active (10/10); artery, hypertrophy, medial (10/10); artery, thrombus (3/10); bronchiole, epithelium, hyperplasia (10/10); BALT, hyperplasia, lymphocyte (9/10); infiltration cellular, histiocyte (10/10); metaplasia, goblet cell (10/10)	<u>Larynx:</u> epiglottis, metaplasia, squamous (3/9)	<u>Larynx:</u> epiglottis, metaplasia, squamous (7/10) <u>Lung:</u> perivascular, inflammation, chronic-active (10/10); artery, hypertrophy, medial (10/10); artery, thrombus (1/10); bronchiole, epithelium, hyperplasia (10/10); BALT, hyperplasia, lymphocyte (9/10); infiltration cellular, histiocyte (10/10); metaplasia, goblet cell (10/10) <u>Bronchial lymph node:</u> hyperplasia, lymphocyte (2/8)

Genetic Toxicology

Micronucleated Erythrocytes (In Vivo):   Negative in males and females

BALT = bronchus-associated lymphoid tissue.
[a]None = no toxicologically relevant effects for this endpoint.

Introduction
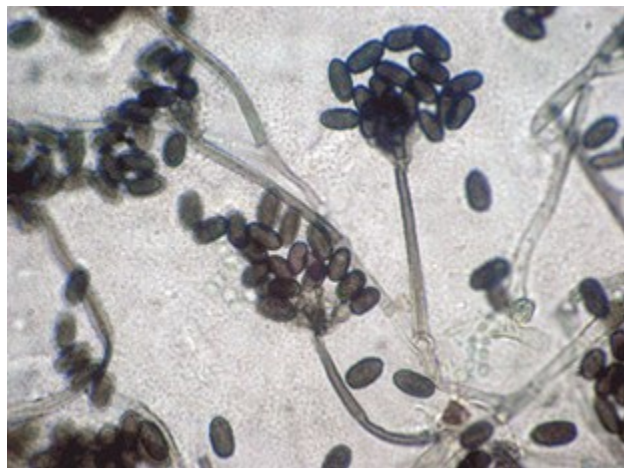
Figure 1. *Stachybotrys chartarum* (CASRN 67892-26-6)

Botanical name: *Stachybotrys chartarum*. Synonyms: *Stachybotrys chartarum (S. chartarum)*; *Stachybotrys atra (S. atra)*; *Stachybotrys alternans (S. alternans)*; *Stachybotrys chartarum (Ehrenb.)* S. Hughes 1958; anamorphic, satratoxin-producing chemotype (CBS 414.95; IBT 9460).[a] The light microscopic image depicts asexual *S. chartarum* reproductive structures composed of septate hyphae, conidiophores, and conidiospores.
Image courtesy of Mold & Bacteria Consulting Laboratories.[2]

Fungi, Indoor Environmental Quality, and Public Health

Fungi are a diverse kingdom with an estimated 5.1 million species.[3] These eukaryotic organisms contain membrane-bound organelles with a rigid cell wall composed of mannose proteins, complex polysaccharides such as α/β glucans, chitin, and ergosterol.[4; 5] The lifecycles of fungi can be unicellular yeast, multicellular hyphae, or a combination of the two (dimorphism). Fungi reproduce asexually or sexually and produce spores, which can also be produced by a variety of other organisms including plants and bacteria. Conidia are asexual fungal spores that are morphologically distinct, ranging from 2 to >120 µm in size.[4] Fungi are mostly saprophytic heterotrophs that obtain nutrients from decaying organic material, allowing growth in environments ranging from soil, agricultural commodities, and indoor environments containing cellulose-based building material. Although most fungal species are not pathogenic, some cause disease in plants, animals, and humans, and the potential for adverse health effects following fungal exposure has become an area of public concern in the United States.

Damp indoor environments may provide optimal growth conditions required for fungal proliferation. Abiotic or biotic disturbances can result in the aerosolization of fungal bioaerosols composed of spores, filamentous hyphae, or microscopic fragments into the surrounding environment. Exposure can also occur in occupational settings, including forestry, agriculture, service, and manufacturing, and can consist of spore concentrations exceeding 10^8 colony-forming units per cubic meter (CFU/m^3).[6] Along with spores, hyphal fragments can also be aerosolized into the breathing zone of exposed individuals and can be found at higher concentrations than spores.[7-12] Fungal bioaerosols can contain secondary microbial metabolites,

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[a]IBT 9460 is an anamorphic, satratoxin-producing *S. chartarum* chemotype (chemotype S) originally isolated from a water-damaged building in Finland.[1]

such as mycotoxins and microbial volatile organic compounds (MVOCs), that have the potential to further exacerbate the negative health responses following exposure.

A study of water-damaged building materials collected from damp indoor environments (n = 72) identified fungal genera including *Penicillium* (68%), *Aspergillus* (56%), *Chaetomium* (22%), *Ulocladium* (21%), *Stachybotrys* (19%), and *Cladosporium* (15%), all of which are known to cause respiratory allergy.[13] Of these six commonly identified fungi, *Stachybotrys chartarum* was one of the most frequently detected fungal species. In 1994, evidence of acute idiopathic pulmonary hemosiderosis (IPH) was identified in infants residing in Cleveland, OH.[14; 15] Further investigation suggested that more of the infants diagnosed with acute IPH resided in water-damaged homes than in homes that did not contain water damage, and *S. chartarum* was identified as a potential causative agent.[15] However, a later report published by the Centers for Disease Control and Prevention refuted those results, stating that the evidence presented in the initial report did not support the epidemiological associations between water damage, *S. chartarum* exposure, and acute IPH.[15] Although the initial association between human illness and mold contamination was nullified, awareness of the association between fungal exposure and human health was already heightened.

Following the Cleveland investigation, consensus documents from the Institute of Medicine, Committee on Damp Indoor Spaces and Health,[16] and the World Health Organization[17] identified epidemiological evidence of associations between exposure to mold contamination in damp indoor environments and adverse respiratory symptoms such as asthma, dyspnea, wheeze, cough, respiratory infections, bronchitis, allergic rhinitis, eczema, and upper respiratory tract symptoms.[18] In addition, fungal exposure has been associated with allergic bronchopulmonary mycoses and hypersensitivity pneumonitis.[6; 19] In 2017, cost estimate analyses indicated that the direct health-care costs of all fungal-related diseases exceeded $7.2 billion, which included $4.5 billion from hospitalizations and $2.6 billion from outpatient visits.[20] Although associations between mold contamination in damp indoor environments and adverse health effects have been identified, the pulmonary and systemic toxicity resulting from repeated fungal exposure remains largely unknown. In addition, there are no current exposure limits or regulations in place to protect individuals who encounter contaminated fungal environments.

To address these knowledge gaps, fungi were nominated to the National Toxicology Program (NTP) in May 2004. The NTP Board of Scientific Counselors and Executive Committee suggested studying fungal species commonly identified in indoor environments, including *Aspergillus*, *Alternaria*, and *Stachybotrys*. In 2007, an expert review panel discussed the NTP subchronic study design, including the fungal test article production, characterization, and reproducibility of the test article generation. As a participant of the review panel, the National Institute for Occupational Safety and Health (NIOSH) was identified as a collaborative partner to conduct subchronic (13-week) inhalation studies to examine the toxicological effects following repeated fungal exposure. To complete these subchronic inhalation exposures, NIOSH developed a computer-controlled acoustical generator system (AGS) to deliver dry aerosolized fungal test articles to mice housed in nose-only chambers to model a natural human inhalation exposure that would occur in residential or occupational environments. The use of the AGS circumvents limitations of other administration methods, including intranasal exposure,[21] intratracheal instillation,[22-25] and liquid aerosol inhalation.[26] Environmentally and occupationally relevant fungal species nominated to NTP have been independently tested in both pilot immunology

studies conducted by NIOSH[27-29] and in toxicology studies reported by NTP in collaboration with NIOSH.[30]

A 4-week and 13-week pilot exposure study using the AGS evaluated the pulmonary immune responses to repeated *S. chartarum* exposure.[27] Following the initial optimization and characterization of the *S. chartarum* test articles, including a heat-inactivated biological particle control,[28] groups of B6C3F1/N female mice repeatedly inhaled one of two viable trichothecene-producing strains of *S. chartarum* twice a week for a total of 4 or 13 weeks.[27] The two *S. chartarum* strains used in the pilot study were IBT 9460 and IBT 7711. IBT 9460 is a macrocyclic trichothecene-producing strain that produced a higher level of mycotoxin and fragmented to a greater extent than IBT 7711.[28] Separate groups of mice inhaled either nonviable *S. chartarum* conidia (a heat-inactivated particle control for each respective strain) or high-efficiency particulate air [filter] (HEPA)-filtered air only. Pulmonary arterial remodeling, characterized by airway inflammation with a granulocyte infiltration, was observed after 4 weeks of *S. chartarum* IBT 9460 exposure. Granulocyte infiltration switched to more of a lymphocytic and macrophage infiltration after 13 weeks of exposure to both IBT 9460 and IBT 7711. Perivascular inflammation and bronchiolar epithelial cell hyperplasia were also observed. Flow cytometry analysis of bronchoalveolar lavage fluid (BALF) from exposed mice showed an increase in eosinophils, neutrophils, and macrophages following a 13-week exposure to both IBT 9460 and IBT 7711. However, the progression of cellular infiltrates and immunopathological responses progressed more rapidly in IBT 9460-exposed mice compared to IBT 7711-exposed mice, suggesting that the presence of fungal fragments plays a critical role in the pulmonary immune response to *S. chartarum* exposure.

In an NTP study conducted in collaboration with NIOSH describing the toxicological responses following a subchronic *A. fumigatus* exposure, the incidence of nonneoplastic lesions was significantly increased in the larynx, lung, and bronchial lymph nodes of viable *A. fumigatus*-exposed male and female B6C3F1/N mice compared to the control groups.[30] In the lung, arterial medial hypertrophy was identified and described as circumferential thickening of the tunica media of small and medium arterioles and arteries by concentric layers of hypertrophied and hyperplastic smooth muscle cells. In addition, chronic-active inflammation, defined as the increased presence of neutrophils, was observed in the lung tissue following subchronic exposure to *A. fumigatus*. Bronchus-associated lymphoid tissue (BALT) hyperplasia and goblet cell metaplasia were also observed following *A. fumigatus* exposure. Squamous metaplasia of the epiglottis was observed in the larynx of both male and female mice exposed to viable *A. fumigatus* compared to the heat-inactivated particle control and air control groups. Evaluation of the bronchial lymph nodes showed significantly increased incidences of lymphocyte hyperplasia and plasma cell hyperplasia relative to either the heat-inactivated particle control or air control groups.[30]

The purpose of these studies on *A. fumigatus* was to characterize the toxicological and pulmonary immunological responses following repeated exposure to fungal contaminants commonly identified in occupational and damp indoor environments. Using a method that replicates a more natural human exposure has provided unique opportunities to define the mechanisms by which these fungal species cause adverse respiratory health effects. To further understand the hazards following *S. chartarum* exposure, NIOSH employed this same methodology to characterize the toxicological responses following subchronic exposure to

S. chartarum strain IBT 9460, a high trichothecene-producing, high fragmenting strain of *S. chartarum*.

Chemical and Physical Properties

The genus *Stachybotrys* is an asexually reproducing, filamentous fungal genus that includes 50 species found worldwide and belongs to the Ascomycota order Hypocreales, family Stachybotryaceae.[31] *S. chartarum* is a macroscopic saprophyte that is greenish black in color, leading to the common name "black mold." This fungus was first identified in 1818 as *Stillbospora chartarum* Ehrenb.[32] In 1837, August Carl Joseph Corda identified the fungus as *Stachybotrys atra* following collection from wallpaper in a house in Prague,[33] which was then reclassified as *S. chartarum* (Ehrenb.) S. Hughes 1958.[34] *S. chartarum* requires a cellulose-rich substrate, such as drywall, wallpaper, gypsum board, or cardboard, and high moisture content for optimal growth.[35-37] *S. chartarum* can grow at a range of temperatures, but the most optimal condition for growth is 30°C at 93%–94% humidity.[38] Frazer and colleagues[39] showed different temperature and water activities are required for the germination and growth of *S. chartarum* compared to sporulation. *S. chartarum* is often found in damp indoor environments.[1; 11; 40-42] Consequently, one study comparing water-damaged building materials collected from damp indoor environments (n = 72) identified *S. chartarum* more often than other fungal species such as *Penicillium chrysogenum* and *A. versicolor*.[13]

The mycelium of *S. chartarum* is characterized by the formation of dark, slimy masses of conidia (spores) that sporulate abundantly from phialides. The phialides are 9–14 mm in length and often occur in whorls that extend from the tip of conidiophores connected to septate hyphae. *S. chartarum* conidia are smooth or ridged, ellipsoid, and unicellular and range from 7 to 12 µm in length and 4 to 6 µm in width.[43] The aerodynamic diameter of aerosolized *S. chartarum* (strain IBT 9460) conidia has been reported to be approximately 3–5 µm[28]; however, one study showed that North American strains have an average aerodynamic diameter of 5.6 µm, but the conidia size was variable, and approximately one-third of the conidia tested were within the respiratory range.[43] This high variability in conidia size could potentially be due to the presence of fungal fragments, a notion supported by aerosolization studies describing the high occurrence of fungal fragments. One study conducted by Lemons et al. used *S. chartarum* strain IBT 9460 and showed that acoustical generation resulted in the aerosolization of not only intact *S. chartarum* conidia (3–5 µm) but also of fungal fragments (<2 µm aerodynamic diameter) derived from conidia, phialides, and hyphae that initially constituted 50% of the total fungal particle count.[28] Cho et al. conducted a separate study, which found that *S. chartarum* fungal fragments were aerosolized at concentrations 514 times higher than *S. chartarum* spores.[8]

It has been suggested that the mycotoxins produced by *S. chartarum* contribute to the adverse health effects observed following exposure.[22; 37; 44-46] Two chemotypes of *S. chartarum* exist, chemotype S and chemotype A, and are defined by the mycotoxins produced. Used for this current study, chemotype S produces toxic macrocyclic trichothecenes including satratoxins, roridins, and verrucarins,[47; 48] whereas chemotype A produces less toxic atranones.[1; 49] In addition, *S. chartarum* also produces phenylspirodrimanes,[41] trichoverrols,[50] trichoverrins,[50] and spirocyclic drimanes,[1] as well as MVOCs,[51] which individuals could encounter within *S. chartarum*-contaminated environments.

Human Exposure

S. chartarum is distributed worldwide and is found in soil and on cellulose-rich materials including hay, straw, plant debris, and building materials.[35; 37; 52] Human exposure to contaminated materials, such as straw contaminated with *S. chartarum*, has resulted in dermatological and adverse respiratory health effects.[53; 54] Exposure to *S. chartarum* can occur via dermal contact, ingestion, and inhalation; however, studies have shown that exposure occurs primarily through direct skin contact and inhalation.[44; 54] *S. chartarum* spores do not readily disperse into the air because of the slime droplet encasing the phialides but can become aerosolized when the fungus dries and is disturbed via biotic or abiotic air movements.[44] Exposure to fungal bioaerosols, which include *S. chartarum*, generated from disturbances within fungal-contaminated, water-damaged environments have been associated with poor indoor air quality[11; 48] and adverse respiratory health effects.[16; 17]

Fungal bioaerosols can be composed of spores, filamentous hyphae, and microscopic fragments. Fungal fragments have the potential to aerosolize at higher concentrations than spores.[7-12] In addition, certain strains of *S. chartarum* fragment to a greater extent compared to other *S. chartarum* strains or fungal species[11; 28] and may contribute to the adverse respiratory outcomes following *S. chartarum* exposure.[27] Case reports have identified an association between indoor *S. chartarum* exposures and respiratory symptoms,[55-57] immunological dysfunction,[58] and neurological disease; however, there is a lack of evidence for neurotoxicity.[41]

S. chartarum has been detected in water-damaged environments such as schools, offices, and home dwellings[13; 58-61] and has been associated with "sick building syndrome" and damp-related building illness.[44; 46; 59; 62-66] Workers remediating *S. chartarum*-contaminated material from buildings reported symptoms, including throat irritation, fatigue, muscle aches, and skin rashes.[57] Sampling of contaminated work areas resulted in an estimated air concentration of 2×10^4 CFU/m^3,[58; 67] which coincided with other measured *S. chartarum* airborne levels reported to be 7×10^5 viable conidia/m^3.[43] A case control study of 10 infants with pulmonary hemorrhage from Cleveland, OH reported the mean air concentration of *S. atra* (synonym *S. chartarum*) was 43 CFU/m^3 compared to 4 CFU/m^3 in the homes of infants without pulmonary hemorrhage.[68] Residents living in water-damaged homes have shown severe asthma with an allergic reaction to *Stachybotrys* antigens isolated from moldy carpets.[69] Neurological and flu-like symptoms, fatigue, dermatitis, and malaise have also been reported from a home with identified *Stachybotrys* contamination, symptoms of which resolved following the removal of *Stachybotrys*-contaminated material.[55] Several studies that have characterized the responses of individuals exposed to *S. chartarum* suggested that the symptoms reported were likely caused by inhalation of the fungal toxins.[56; 58; 61]

S. chartarum is considered nonpathogenic.[70] The ability of the species to contribute to adverse health outcomes is suggested to be caused by the allergenicity, inflammatory response, or toxicity of *S. chartarum*. It was once thought that *S. chartarum* was incapable of infection because of the lack of physical evidence of the organism within a symptomatic individual. Most recently, however, *Stachybotrys* (*S. chlorohalonata*) was identified in the sinus tissues of a severely immunocompromised 23-year-old man.[71] Although mycotoxins were also reported to contribute to the hemorrhaging observed in the immunocompromised male patient, this was the first report of an invasive case of *Stachybotrys*.

Regulatory Status

Currently, there are no established guidelines for acceptable *Stachybotrys* concentrations in residential or occupational environments. In addition, no exposure limits have been proposed by any regulatory or governmental body.

Toxicity

S. chartarum has been characterized as a toxigenic mold that can cause animal and human mycotoxicosis.[41] The effect of *S. chartarum* trichothecenes was identified in the 1930s Russian equine outbreak when horses died after consuming *S. chartarum*-contaminated straw and grain.[52] This newly found disease caused by ingesting or handling contaminated feed was termed stachybotryotoxicosis and has been reported worldwide in animals and humans.[43; 53; 72] Farm workers exposed to *S. chartarum*-contaminated hay complained of dyspnea, airway obstruction, conjunctivitis, sore throat, mucosal irritation, and skin inflammation.[73] Previous studies suggest that the occupational exposure concentrations resulting in trichothecene toxicosis were on the order of 10^3–10^7 conidia/m^3.[43] Although these mycotoxicosis cases were due to direct skin contact or ingestion, inhaling mycotoxins is considered more harmful because of the greater fungal bioavailability.[43]

Distinct mycotoxin profiles are produced by *S. chartarum* when grown on different culture media.[74] However, research has indicated that the water activity (a_w) must reach a level of 0.95 a_w before mycotoxins are produced.[48] *S. chartarum* produces macrocyclic trichothecenes, such as satratoxins and isosatratoxins F, G, and H, roridin E and L-2, and verrucarins J and B. These macrocyclic trichothecenes are suggested to contribute in part to the adverse health effects following *S. chartarum* exposure.[22; 37; 44-48] Research has shown that *S. chartarum* macrocyclic trichothecenes within indoor contaminated environments can become airborne and are associated with both conidia[75] and fungal fragments.[76; 77] Mycotoxins have been associated with particle sizes ≥3 µm, which correspond to conidia; however, trichothecene mycotoxins, such as satratoxin H and verrucarin J, have been shown to be associated with other *S. chartarum* components, including submicron fragments that can be inhaled and deposited deep within the lung.[78]

In general, satratoxins have been reported to be produced in larger amounts compared to the other trichothecenes.[72] In mice, the median lethal dose (LD_{50}) for satratoxin has been reported to be approximately 1 mg/kg.[79] Following the aerosolization of *S. atra* (syn. *S. chartarum*) conidia, Sorenson et al. confirmed the presence of satratoxin H within the bioaerosol, as well as satratoxin G and trichoverrols A and B to a lesser extent.[80] Satratoxin G has been reported to be the most cytotoxic of the satratoxins and five other trichothecenes studied.[81] A single dose of ≥25 µg/kg satratoxin G administered via intranasal instillation resulted in neurotoxicity and inflammation within the nose and brain of female C57Bl/6 mice.[82] Isosatratoxin F exposure altered the phospholipid synthesis, composition, and secretion of pulmonary surfactant in BALF in mice[83] and resulted in cytological alterations within fetal rabbit alveolar type II cells, indicative of cellular damage and apoptosis. Additionally, satratoxin-positive *S. chartarum*, as well as the trichothecene mycotoxins (roridin A, verrucarin A, and T-2 toxin), have been shown to induce apoptosis and inflammation in human primary macrophages.[84]

Stachylysin, a hemolysin released from *S. chartarum*,[85-87] was thought to contribute to the infant pulmonary hemosiderosis cases reported in Cleveland, OH.[14; 15; 88] To investigate this potential contribution, a study conducted by Vesper and Vesper[88] measured the production of stachylysin from the *S. chartarum* strains isolated from infant pulmonary hemosiderosis case houses in Cleveland, OH[46] and in an isolate from the lung of a pulmonary hemosiderosis patient in Texas.[89] Results showed the isolated *S. chartarum* strains produced stachylysin, suggesting a potential role in the reported pulmonary hemosiderosis cases.[88]

Exposure to atranones A and C has also been shown to result in immunotoxic and inflammatory responses in the lungs of male Swiss Webster (CFW) mice following a single dose of ≥2 µg/animal via intratracheal instillation.[90] Rand et al. suggested that different atranones exhibit varying levels of inflammation with different toxicokinetics.[90] Another study showed that a single exposure to a nontoxin-producing strain of *S. chartarum* could induce airway irritation and elicit an immune response in male Swiss Webster (CFW) mice.[26]

Fungi also produce MVOCs, and similar to mycotoxins, the production of MVOCs depends on the growth media. For example, MVOCs unique to *S. chartarum* include 1-butanol, 3-methyl-2-butanol, 3-methyl-1-butanol, and thujopsene and have been detected on rice inoculated with *S. chartarum*, while 1-butanol was detected on inoculated gypsum board.[51] MVOCs emitted from contaminated fungal environments have additionally been implicated in central nervous system symptoms such as headaches, inability to concentrate, or dizziness.[91]

Research surrounding the toxic effect of exposure to spores, secondary metabolites, or MVOCs of *S. chartarum* has been confounding. It has been predicted that the adverse health effects observed in animal models are caused by exposure to high spore or mycotoxin concentrations that exceed those encountered in even the most heavily contaminated environments.[37; 92] However, the pediatric IPH cases identified in Cleveland, OH[15] occurred after exposure to fungal-contaminated environments, suggesting that the spore or mycotoxin concentrations encountered in these damp indoor environments were sufficient to cause adverse health effects in children. Additionally, the length of fungal growth and substrate availability influenced the secondary metabolite profiles and the associated effect on adverse respiratory health effects.[93] Regardless of the variability in toxin production, the results of most toxicological studies demonstrate that *S. chartarum* produces highly toxic trichothecenes that have been shown to contribute to adverse health effects.

Reproductive and Developmental Toxicity

While not well studied, one report found that CFW male and female mice orally exposed to a single, low dose (3,000–4,000 tissue culture units) of *S. alternans* (syn. *S. chartarum*) prepared from grain or liquid medium infected with toxicogenic and nontoxicogenic strains resulted in a decreased number of pregnancies compared to control mice.[94] In addition, mice from that same study that had ingested 100–4,000 tissue culture units of toxin had a significant increase in the frequency of dead, resorbed, or stunted fetuses compared to controls.

Carcinogenicity

Several studies have evaluated the carcinogenicity of individual mycotoxins; however, no studies evaluating carcinogenicity following *S. chartarum* exposure were identified.

Genetic Toxicity

Although studies evaluating the effect of purified mycotoxin exposure on genotoxicity exist, no studies evaluating the genetic toxicity following *S. chartarum* exposure were identified.

Study Rationale

Following the nomination of fungi to NTP for comprehensive toxicity evaluation, NTP partnered with NIOSH to begin a series of studies to characterize the toxicological responses following exposure to NTP-nominated fungal species. The first of the series examined the toxicological responses to *A. fumigatus*[30] following delivery of dry aerosolized fungal test articles twice a day for 13 weeks to male and female B6C3F1/N mice housed in nose-only chambers. The design of this inhalation study was to simulate a more natural exposure similar to what would be encountered in a water-damaged built environment. In this second inhalation toxicity study, *S. chartarum* was selected as the test article because of the heightened awareness of adverse health effects of fungal exposures encountered within indoor water-damaged environments. Although pulmonary immunological endpoints have been evaluated in pilot studies, the toxicological responses to *S. chartarum* exposure have not been fully characterized.

Materials and Methods

Procurement and Characterization

Culture of *Stachybotrys chartarum*

Stachybotrys chartarum (CBS 414.95, IBT 9460) was originally isolated from a water-damaged building in Finland.[1] This is a macrocyclic trichothecene-producing strain and can fragment to a greater extent than other *S. chartarum* strains.[28] Bulk *S. chartarum* cultures were cultivated from 25% glycerol stocks prepared as described in Appendix A. Aliquots of the glycerol stock were stored at −80°C for the study duration.

Individual glycerol stock aliquots were used weekly to inoculate two malt extract agar (MEA) plates for the cultivation of *S. chartarum* according to the methods detailed in Appendix A. After 12–14 days of culture, *S. chartarum* conidia (spores) were liberated from each plate using 2 mL of sterile water. Twelve culture flasks containing wet, autoclaved white rice (30 g) were inoculated with 1.25×10^6 conidia. Following 21–28 days of growth, half of the flasks were heat-treated at 80°C for 2 hours to produce the heat-inactivated particle control. The remaining flasks served as the viable test article. The heat-inactivated particle control and viable *S. chartarum* flasks were transferred to the National Institute for Occupational Safety and Health (NIOSH) inhalation facility and placed in a desiccator for 7–10 days prior to aerosolization.

Quality Assurance

Quality assurance and quality control of heat-inactivated and viable *S. chartarum* consisted of testing cultures each week using internal transcribed spacer gene sequencing. This method allowed the amplification and detection of potentially contaminating mycobiota sources as described in Appendix A. Cultures were also tested weekly to ensure reproducibility and production of nonviable *S. chartarum*. All identified fungal DNA belonged to *S. chartarum* with no contaminating fungi detected in the viable or heat-inactivated *S. chartarum* cultures. Heat inactivation resulted in an average of 98.63% reduction in *S. chartarum* (Table A-1).

Test Article Generation and Exposure System

NIOSH has developed a computer-controlled acoustical generator system (AGS), in which acoustical energy is used to release the fungal spores into a nose-only exposure chamber. Figure 2 shows the AGS based on a modified acoustically powered particle (Pitt-3) generator, which was originally described by Weyel et al.[95] The Pitt-3 generator was purchased from the University of Pittsburgh and evaluated under conditions recommended by its engineering school.[96] The system was then fitted with a computer controller, further modified to its current design,[97; 98] and used by NIOSH to study the respiratory effects caused by the inhalation of a wide variety of occupationally relevant dusts. The AGS was initially used as a new and improved system for dispersing nanomaterials[99] and was subsequently modified for fungal spore aerosolization as described by Buskirk et al.[100] Fungi were grown on rice as described above, placed on the rubber membrane of the AGS, and aerosolized using acoustical energy. Inhalation of fungal spores simulates natural fungal exposures in the environment better than other routes of exposure. Several studies conducted by NIOSH to date using the AGS demonstrated that fungal aerosols can be reproducibly administered to mice housed in the nose-only exposure chamber,[27-

[29; 100-102] including a National Toxicology Program (NTP) study assessing the toxicological response to *Aspergillus fumigatus*.[30]
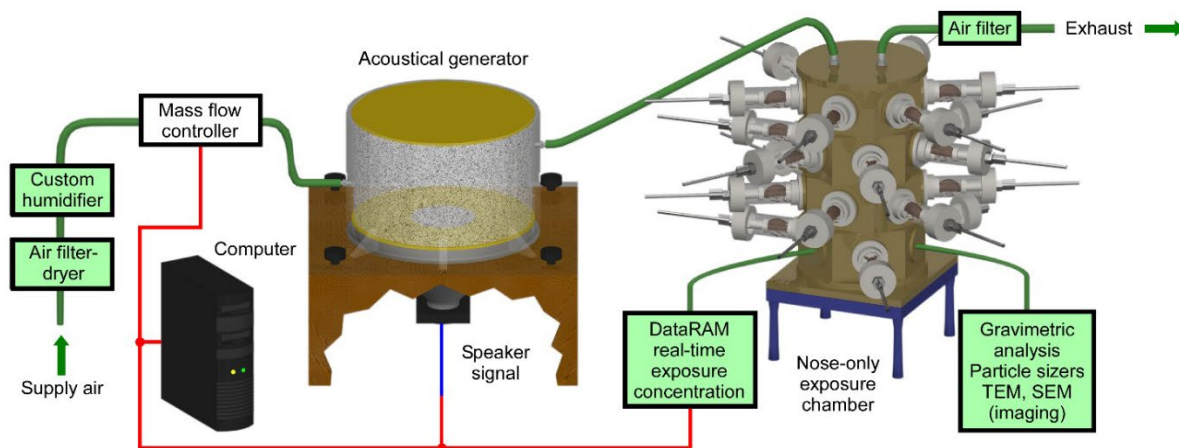
Figure 2. Illustration Showing the Acoustical Generator Nose-only Exposure System

TEM = transmission electron microscope; SEM = scanning electron microscope. High-efficiency particulate air-filtered supply air is directed into the acoustical generator, which vibrates at a designated frequency to form fungal spore aerosols from the fungus-coated rice grains. The fungal aerosol is directed into a nose-only chamber, and then the air is filtered before being sent into the exhaust system. A light-scattering instrument (DataRAM4; ThermoElectron Co., Franklin, MA) attached to the computer estimated the real-time aerosol mass concentration. This value was multiplied by a scale factor to determine the real-time number of spores deposited in the mouse lung based on the previously measured aerosol size distribution and a custom lung-deposition model. The software used that value to alter the amplitude of the speaker signal during exposures to obtain the desired chamber mass concentration.

Initial aerosolization studies demonstrated that the AGS produced an *S. chartarum* aerosol composed of single conidia of similar size (3–5 µm) and morphology in both the viable test article (*S. chartarum*) and heat-inactivated particle control groups (Figure 3). However, unlike the heat-inactivated particle control, a significant proportion of the viable *S. chartarum* aerosol was also composed of smaller fragments (<2 µm, Figure 3A).[28]
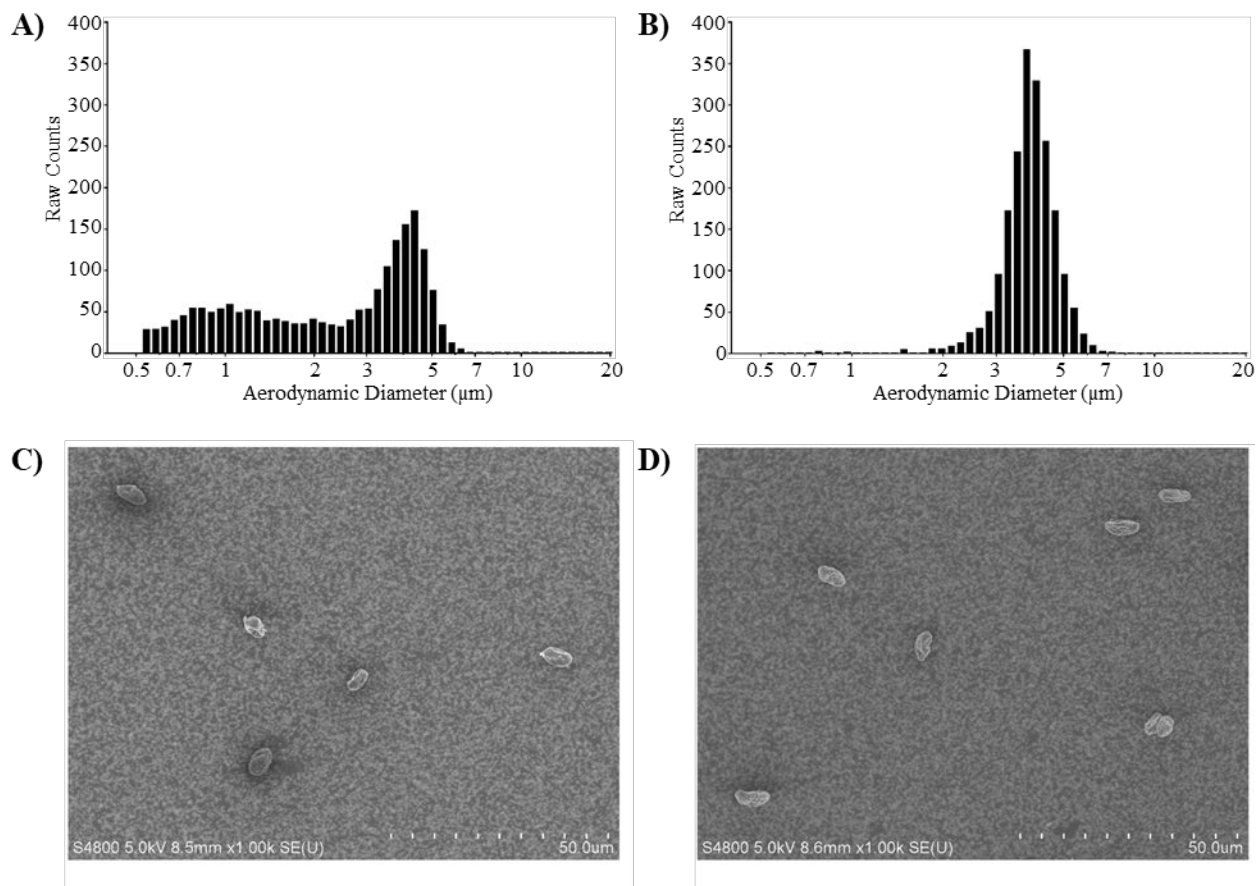
Figure 3. Aerodynamic Particle Size Measurements and Electron Microscopy Images Captured Following Aerosolization of *Stachybotrys chartarum* Conidia

Top panel images depict the size distribution of fungal particles measured with the aerodynamic particle sizer following aerosolization of (A) the test article, viable *S. chartarum* conidia, and (B) the heat-inactivated particle control, nonviable *S. chartarum* conidia. Bottom panel images display field emission scanning electron microscopy images of conidia collected on filters following acoustical generator system (AGS) aerosolization of (C) the test article conidia and (D) the heat-inactivated particle control. Images demonstrate that both groups result in an aerosol consisting of single conidia of similar size (3–5 µm), as well as the smaller fungal fragments seen only in the test article group.

Aerosolized viable *S. chartarum* and the heat-inactivated particle control were delivered to male and female B6C3F1/N mice housed in a multianimal nose-only exposure chamber via the AGS as previously described.[27; 28] Animals were exposed two times per week for 3 months via inhalation. In addition, an air control group received the same high-efficiency particulate air [filter] (HEPA)-filtered air that was being used to deliver viable *S. chartarum*.

Inhalation exposures were achieved by weighing and then placing the mice in a custom-built nose-only multianimal exposure carousel connected to the AGS as described in McKinney et al.[99] Mice were placed in random pods for each exposure to minimize the effects of slight concentration differences observed within individual ports. Although the nose-only carousel houses up to 24 pods (Figure 2), only 20 pods (10 male and 10 female mice) were used during each exposure. Additional ports served as sample ports for the collection and analysis of test articles. The air control, heat-inactivated particle control, and viable *S. chartarum*-exposed animals were exposed using different chambers located in the same inhalation exposure room.

B6C3F1/N mice were exposed to viable *S. chartarum* or heat-inactivated particle control until the DataRAM software estimated that a total of approximately 1×10^4 conidia had been deposited within the lungs during the 1-hour exposure session. Figure 4 shows the estimated pulmonary deposition following each exposure for the duration of the study. The estimation software used a mouse-lung-deposition model developed by the NIOSH Inhalation Facility, which was based on deposition measurements from Raabe et al.[103] During viable *S. chartarum* or heat-inactivated particle control exposures, lung-deposition calculations were achieved using real-time DataRAM mass concentration measurements and a previously described scaling factor that estimated the number of conidia deposited in the lungs of mice in real time.[100; 102] The integrated value of mass deposition per unit time was calculated throughout the exposures. The AGS automatically turned off when the desired total conidia deposition had been delivered to the mice; however, the mice continued to receive HEPA-filtered humidified (approximately 40% relative humidity) air supplied at a flow rate of 6 L/min until 60 minutes had been reached. Each exposure corresponded to a 60-minute average mass concentration of approximately 8 mg/m³ (viable *S. chartarum*) or 7 mg/m³ (heat-inactivated particle control). Gravimetric filters were also collected during each exposure to continually monitor and adjust the DataRAM calibration. Air control mice received only HEPA-filtered air supplied to the chamber at the same flow rate (6 L/min) and time (60 minutes) as *S. chartarum*-exposed mice. All environmental conditions were kept constant in each carousel between control and test article animals.
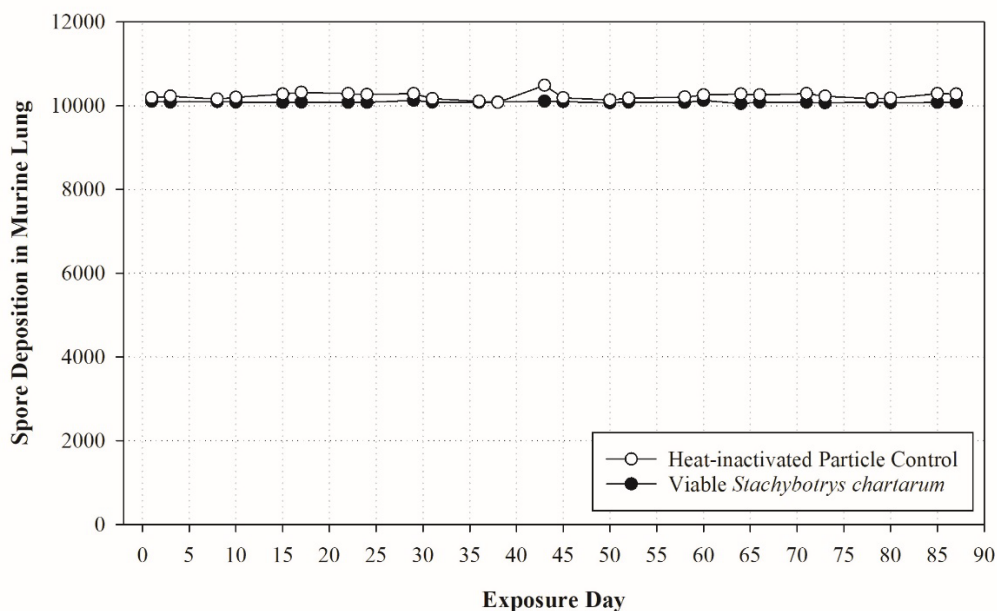
Figure 4. Estimated Pulmonary Deposition of *Stachybotrys chartarum* Conidia for Each Exposure over the Course of the Three-month Study

Values represent the number of conidia estimated to deposit in the lung for viable *S. chartarum* (solid circles) and heat-inactivated particle control (open circles) exposure groups.

Animal Source

Male and female B6C3F1/N mice (n = 20/exposure group and 20 sentinel mice) were obtained from the NTP colony maintained by Taconic Biosciences, Inc. (Germantown, NY).

Animal Welfare

Animal care and use were in accordance with the Public Health Service Policy on Humane Care and Use of Animals. The NIOSH Inhalation Facility is an environmentally controlled barrier facility that is fully accredited by AAALAC International. Studies were approved by the CDC-Morgantown Animal Care and Use Committee and conducted in accordance with all NIOSH animal procedures and policies.

Exposure Concentration Selection Rationale

An estimated pulmonary dose of 1×10^4 *S. chartarum* conidia (viable or heat-inactivated conidia) was chosen for this study. This concentration was the highest aerosolized dose that reproducibly induced a lymphoproliferative response and was the same concentration and schedule used for a previously published pulmonary immunology study.[27; 28] This estimated lung burden would be equivalent to that of a worker breathing a constant workplace air concentration of 3.45×10^6 conidia/m^3 (146 μg/m^3) for 1 week, working 8 hours per day for 5 days, and is comparable to an occupational exposure concentration of 10^3–10^7 conidia/m^3, which has been reported to result in toxicosis.[43]

Three-month Study

Mice were approximately 5 to 6 weeks old on receipt. The animals were weighed, distributed randomly into groups of approximately equal initial mean body weights, and randomly assigned to viable *S. chartarum*, heat-inactivated particle control, or air control groups. Mice were initially identified via tail marking, using a permanent marker and a consecutive numbering system.

Animals that weighed >10% or <10% of the mean of the group were placed in the sentinel group (n = 20). At 4 weeks and at study termination, serological analyses for bacterial and viral pathogens were performed on 10 male and 10 female sentinel mice using the protocol of the NIOSH Sentinel Animal Program (Appendix C).

Before the study, the mice were acclimated to the NIOSH animal facility for a minimum of 7 days. Afterward, to reduce potential stress from exposures, the mice were acclimated to the housing units used for the exposures (nose-only carousel) for 1 week (two 1-hour acclimations) before the initial fungal exposure. Study mice were placed individually in the carousel housing units (pods), and humidified HEPA-filtered air was supplied to the chamber for 1 hour on 2 separate days during the week before the initial exposures. After acclimation was complete and before the exposures began, the mice were tattooed using a consecutive numbering system.

Male and female B6C3F1/N mice were randomly assigned to viable *S. chartarum*, heat-inactivated particle control, and air control groups. Using a modified NTP protocol, groups of 10 male and 10 female mice were placed in individual pods on the carousel on the same 2 days of the week for 26 exposures (3 months). The mouse positions in the carousel were rotated throughout the study to account for any dose variability based on pod placement. Male and female mice were exposed for 1 hour per day to 8 mg/m^3 (resulting lung dose = 1×10^4) viable *S. chartarum* conidia, 7 mg/m^3 (resulting lung dose = 1×10^4) heat-inactivated *S. chartarum* conidia (particle control), or HEPA-filtered air (air control) that was delivered through the AGS into the nose-only exposure chamber at a flow rate of 6 L/min.

Following acclimation and exposures, the animals were transferred from the individual carousel housing pods to a standard cage environment located in an adjoining room within the facility. Male and female mice were housed in HEPA-filtered ventilated polycarbonate cages on autoclaved hardwood chip bedding. Male B6C3F1/N mice were individually housed during acclimation and the in-life phases of the study because of social incompatibilities, whereas female mice were housed in groups of five. Enrichment was provided to all cages and consisted of crinkle cut kraft paper. The room temperature of the animal facility was maintained between 68°F and 76°F, and the relative humidity ranged between 17% and 70% (Table 1). The light/dark cycle was maintained at 12-hour intervals. Mice were provided feed and tap water ad libitum, except when housed in the pods during acclimation or exposures. Sentinel mice selected during the acclimation period were housed within the same room and were free of viral and bacterial pathogens (Appendix C).

Study animals were weighed upon initial receipt, and body weights were recorded weekly until study termination. Clinical observations were recorded daily for the duration of the study, once a day (morning) on nonexposure days and twice a day (morning/afternoon) on exposure days. Information about the study design, facilities, and animal maintenance is provided in Table 1. The report on feed composition and contaminants is provided in Appendix B.

Clinical Examinations and Pathology

At the completion of the 3-month study, the mice were injected intraperitoneally with a lethal dose of sodium pentobarbital euthanasia solution (100–300 mg/kg). Peripheral blood for hematology and micronuclei determination was obtained via cardiac puncture. Whole blood (a minimum of 600 µL) was collected in tubes containing potassium ethylenediaminetetraacetic acid, and 400 µL were immediately processed for hematology using a ProCyte Dx hematology analyzer (IDEXX Laboratories, Inc., Westbrook, ME). Table 1 lists the parameters measured. A minimum of 200 µL of blood was immediately refrigerated and shipped to Integrated Laboratory Systems, LLC (ILS; Research Triangle Park, NC) for micronuclei determination.

Necropsies were performed at the NIOSH site by Battelle staff (Columbus, OH) in random order on all animals surviving to terminal sacrifice. The organs weighed at terminal necropsy included the left and right epididymides, heart, left and right kidneys, liver, lungs, left and right ovaries, left and right testes, and thymus. Bilateral organs were weighed and recorded separately. At necropsy, the tissues in Table 1 were examined in situ for gross lesions, removed, and then fixed and preserved in 10% neutral buffered formalin. Exceptions included the eyes and testes (epididymis and vaginal tunics of testes), which were initially fixed in Davidson's solution and modified Davidson's solution, respectively, then transferred to 10% neutral buffered formalin. The collected and fixed tissues were then shipped to the Battelle facility and further processed and trimmed, embedded in paraffin, sectioned at a thickness of 5 µm, and stained with hematoxylin and eosin (H&E). Histological sections of aorta, gallbladder, heart, liver, lung (all five lobes), bronchial lymph node, and spleen were stained with Grocott's methenamine silver (GMS) stain for fungal elements. Battelle laboratory pathologists conducted a complete histopathological examination on all air control, heat-inactivated particle control, and viable *S. chartarum*-exposed mice. Table 1 lists the tissues and organs that were examined.

Microscopic evaluations were completed by a board-certified veterinary pathologist, and the pathology data were entered into the NTP Provantis software (Instem Stone, UK). The report,

slides, paraffin blocks, residual wet tissues, and pathology data were sent to the NTP Archives for inventory and storage. An audit of pathology specimens was conducted wherein the wet tissues, blocks, and slides were examined by technical staff for quality and adherence to NTP Specifications,[104] and the wet tissues were examined by a team of pathologists to ensure all were sampled according to NTP Specifications. The slide and tissue counts were also verified. Slide-mounted, H&E-stained slides were evaluated by a team of quality assessment (QA) pathologists at a pathology laboratory independent of the study laboratory for accuracy and consistency of diagnoses. The histotechnique was also evaluated.

After a review of the laboratory reports and selected histopathology slides by a QA pathologist, the findings and reviewed slides were submitted to the Pathology Working Group (PWG) coordinator. Any inconsistencies in the diagnoses made by the study laboratory and QA pathologist were resolved by the DTT pathology peer-review process. Final diagnoses for reviewed lesions represent a consensus of the PWG or a consensus between the study laboratory pathologist, DTT pathologist, and QA pathologist/PWG coordinator. Details of these review procedures have been described, in part, by Boorman[105] and Maronpot.[106]

Table 1. Experimental Design and Materials and Methods for the Three-month Inhalation Study of *Stachybotrys chartarum*

Three-month Study
Study Laboratories
National Institute for Occupational Safety and Health (Morgantown, WV) Battelle (Columbus, OH)
Strain and Species
B6C3F1/N mice
Animal Source
Taconic Biosciences, Inc. (Germantown, NY)
Time Held before Studies
19 days
Average Age When Studies Began
8 weeks
Date of First Exposure
April 4, 2016
Duration of Exposure
3 months
Date of Last Exposure
June 29, 2016
Necropsy Date
June 30, 2016
Average Age at Necropsy
21 weeks

Three-month Study

Size of Study Groups

10 males and 10 females

Method of Distribution

Animals were distributed randomly into groups of approximately equal initial mean body weights.

Animals per Cage

1 (male), 5 (females)

Method of Animal Identification

Tail tattoo

Diet

Irradiated NTP-2000 wafers (Zeigler Brothers, Inc., Gardners, PA), available ad libitum, changed at least weekly

Water

Tap water (Morgantown, WV municipal supply) provided in water bottles ad libitum

Cages

HEPA-filtered, ventilated polycarbonate cages housed in a cage rack system (Lab Products Inc., Seaford, DE). Cages that housed three or more mice (females) were changed weekly. Cages that housed two or fewer mice (males) were changed every other week.

Bedding

Autoclaved hardwood chip bedding (Teklad Certified Sani-Chips®; Envigo, Madison, WI) and irradiated crinkle cut kraft paper were provided for nesting/enrichment (Crink-l'Nest™; The Andersons, Inc., Maumee, OH) and changed with cage changes.

Animal Room Environment

Temperature: 71.97°F± 1.41°F (min: 67.72°F, max: 75.78°F)
Relative Humidity: 50.16% ± 11.82% (min: 17.10%, max: 69.27%)
Room fluorescent light: 12 hours/day
Room air changes: 10–15/hour

Test Article

Stachybotrys chartarum (*S. chartarum,* CASRN 67892-26-6) conidia: untreated (viable) or heat-inactivated particle controls
Synonyms: Stachybotrys atra (S. atra); Stachybotrys alternans (S. alternans); Stachybotrys chartarum (Ehrenb.) S. Hughes 1958; anamorphic, satratoxin-producing chemotype (CBS 414.95; IBT 9460)

Exposure Concentrations

Estimated lung depositions:
   Air control: 0 (HEPA-filtered air only)
   Heat-inactivated particle control: 1×10^4 nonviable *S. chartarum* conidia (exposure chamber mass concentration: ~7 mg/m^3)
   Test article: 1×10^4 viable *S. chartarum* conidia (exposure chamber mass concentration: ~8 mg/m^3)

Type and Frequency of Observation

Observed once daily on nonexposure days (morning) and twice daily on exposure days (morning/afternoon). Clinical findings were recorded at each observation and at study termination. Animals were weighed initially and then weekly until study termination.

Three-month Study

Method of Euthanasia

Intraperitoneal injection of a lethal dose of sodium pentobarbital (100–300 mg/kg; Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI) followed by exsanguination via cardiac puncture.

Necropsy

Necropsies were performed on all study mice. Organs weighed were epididymides (left and right), heart, kidneys (left and right), liver, lungs, ovaries (left and right), testes (left and right), and thymus.

Hematology

Blood was collected from all surviving study mice via cardiac puncture at study termination. Hematological analyses included hematocrit, erythrocyte count, reticulocyte count, hemoglobin, mean cell volume, mean cell hemoglobin, mean cell hemoglobin concentration, platelet count, and leukocyte count and differential.

Histopathology

Complete histopathology was performed on all study mice. In addition to gross lesions, the following tissues were examined: adrenal gland, aorta, bone marrow, brain, esophagus, eye, femur, gallbladder, Harderian gland, heart, intestine (large and small), kidney, larynx, liver, lung, lymph nodes (bronchial, mandibular, mediastinal, mesenteric), mammary gland, nose, pancreas, pancreatic islets, parathyroid gland, pituitary gland, salivary glands, skin, spleen, stomach, thymus, thyroid gland, trachea, and urinary bladder. Other male tissues examined included the epididymis, preputial gland, prostate gland, seminal vesicle, and testes. Other female tissues examined included the cervix, clitoral gland, ovary, uterus, and vagina.

HEPA = high-efficiency particulate air.

Statistical Methods

Calculation and Analysis of Nonneoplastic Lesion Incidences

The incidences of nonneoplastic lesions are presented as numbers of animals bearing such lesions at a specific anatomic site and the numbers of animals with that site examined microscopically. Fisher's one-sided exact test,[107] a procedure that uses the overall proportion of affected animals, was used to determine significance between all pairs of the three exposure groups.

Analysis of Continuous Variables

Two approaches were employed to assess the significance of all pairwise comparisons between exposed and control groups in the analysis of continuous variables. Organ and body weight data, which historically have approximately normal distributions, were analyzed using a one-way analysis of variance with post hoc Tukey Honest Significant Difference test[108] for all pairwise comparisons among the three exposure groups. Hematology data, which have typically skewed distributions, were analyzed using the nonparametric multiple comparison method of Dunn.[109] Prior to statistical analysis, extreme values identified by the outlier test of Dixon and Massey[110] were examined by DTT personnel, and implausible values were eliminated from the analyses.

Quality Management

The 3-month study was conducted in compliance with U.S. Food and Drug Administration Good Laboratory Practice Regulations whenever possible.[111] To comply with the NIOSH Health Effects Laboratory Division (HELD) Laboratory Quality Management System, this study was conducted using validated standard operating procedures and performed in accordance with a

CDC-Morgantown Animal Care and Use Committee-approved animal protocol, as well as a HELD Office of the Director-approved study protocol.[112] In addition, gross pathology and histopathology study records were audited retrospectively by an independent QA contractor against study records submitted to the NTP Archives. Separate audits covered completeness and accuracy of the pathology data, pathology specimens, final pathology tables, and a draft of this NTP Toxicity Report. Audit procedures and findings are presented in the reports and are on file at the National Institute of Environmental Health Sciences. The audit findings were reviewed and assessed by DTT and NIOSH staff, and all comments were resolved or otherwise addressed during the preparation of this Toxicity Report.

Genetic Toxicology

The genetic toxicity of *S. chartarum* was assessed by determining the frequency of micronucleated erythrocytes in mouse peripheral blood. The protocol for these studies and the results are given in Appendix D.

The genetic toxicity studies have evolved from an earlier effort to develop a comprehensive database permitting a critical anticipation of a chemical's carcinogenicity in experimental animals based on numerous considerations, including the relationship between the molecular structure of the chemical and its observed effects in short-term in vitro and in vivo genetic toxicity tests (structure-activity relationships). The short-term tests were developed originally to clarify proposed mechanisms of chemical-induced DNA damage, given the relationship between electrophilicity and mutagenicity,[113] and the somatic mutation theory of cancer.[114; 115] Not all cancers, however, arise through genotoxic mechanisms.

Peripheral Blood Micronucleus Test

Micronuclei (literally "small nuclei" or Howell-Jolly bodies) are biomarkers of induced structural or numerical chromosomal alterations formed when acentric fragments or whole chromosomes fail to incorporate into either of two daughter nuclei during cell division.[116; 117] Acute in vivo bone marrow chromosome aberration and micronucleus tests appear to be less predictive of carcinogenicity than the *Salmonella* test.[118; 119] However, clearly positive results in long-term peripheral blood micronucleus tests have high predictivity for rodent carcinogenicity; a weak response in one sex only or negative results in both sexes in this assay do not correlate well with either negative or positive results in rodent carcinogenicity studies.[120] Because of the theoretical and observed associations between induced genetic damage and adverse effects in somatic and germ cells, the determination of in vivo genetic effects is essential to the overall understanding of the risks associated with a particular exposure.

Results

Data Availability

All study data were evaluated. Data relevant for evaluating toxicological findings are presented here. All study data are available in the National Toxicology Program (NTP) Chemical Effects in Biological Systems (CEBS) database: https://doi.org/10.22427/NTP-DATA-TOX-107.[121]

Three-month Study

All female mice survived to the end of the study. One male mouse in the air control group suffered a hind limb wound and was euthanized on day 8 of exposure. All tissues defined in Table 1 were collected from all 10 males and 10 females following necropsy.

Final survival and body weight data are shown in Table 2 and Table 3. Terminal body weights of male and female mice exposed to viable *S. chartarum* conidia (spores) were not significantly different from the air control or the heat-inactivated particle control groups, nor were terminal body weights significantly different between the heat-inactivated particle control and air control groups. The body weight gain among groups of both male and female mice over the course of the study was similar, as illustrated in Figure 5. Clinical observations collected throughout the duration of the study revealed no remarkable findings (Appendix E).

Table 2. Summary of Survival and Body Weights of Male Mice in the Three-month Inhalation Study of *Stachybotrys chartarum*

Study Day[a]	Air Control		Heat-inactivated Particle Control			Viable *S. chartarum*		
	Wt. (g)[b,c]	n	Wt. (g)	Wt. (% of Controls)	n	Wt. (g)	Wt. (% of Controls)	n
1	24.6 ± 0.4	10	25.0 ± 0.4	101.7	10	24.5 ± 0.4	99.7	10
8	24.8 ± 0.4	9[d]	25.1 ± 0.4	101.1	10	24.5 ± 0.4	98.7	10
15	25.8 ± 0.5	9	26.1 ± 0.4	101.1	10	25.4 ± 0.5	98.8	10
22	26.4 ± 0.4	9	26.6 ± 0.5	100.8	10	26.2 ± 0.5	99.5	10
29	26.8 ± 0.4	9	27.3 ± 0.6	101.7	10	27.1 ± 0.5	100.8	10
36	29.2 ± 0.6	9	29.5 ± 0.8	101.0	10	29.0 ± 0.6	99.3	10
43	28.6 ± 0.5	9	29.1 ± 0.7	101.7	10	28.8 ± 0.7	100.6	10
50	29.3 ± 0.6	9	30.0 ± 0.8	102.2	10	29.6 ± 0.7	100.8	10
58	30.4 ± 0.6	9	31.0 ± 0.8	102.2	10	30.7 ± 0.7	101.0	10
64	31.7 ± 0.6	9	31.9 ± 0.8	100.5	10	32.0 ± 0.7	100.8	10
71	32.2 ± 0.5	9	32.7 ± 0.8	101.5	10	32.6 ± 0.7	101.1	10
78	32.9 ± 0.6	9	33.5 ± 0.9	101.8	10	33.4 ± 0.7	101.6	10
85	33.2 ± 0.7	9	33.7 ± 0.9	101.5	10	34.1 ± 0.7	102.8	10
88	33.3 ± 0.6	9	33.4 ± 0.9	101.5	10	33.9 ± 0.8	101.7	10

[a]Study day 1 is the day animals were placed on study.
[b]Statistical analysis performed using one-way analysis of variance with post hoc Tukey Honest Significant Difference test for all pairwise comparisons. No statistically significant findings were noted at $p \leq 0.05$.
[c]Weights shown are mean ± standard error.
[d]One male in the air control group was euthanized on study day 8 due to a hind leg wound.

Table 3. Summary of Survival and Body Weights of Female Mice in the Three-month Inhalation Study of *Stachybotrys chartarum*

Study Day[a]	Air Control		Heat-inactivated Particle Control			Viable *S. chartarum*		
	Wt. (g)[b,c]	n	Wt. (g)	Wt. (% of Controls)	n	Wt. (g)	Wt. (% of Controls)	n
1	21.2 ± 0.5	10	21.3 ± 0.2	100.6	10	21.3 ± 0.3	100.4	10
8	21.5 ± 0.5	10	21.8 ± 0.3	101.4	10	21.6 ± 0.3	100.4	10
15	22.4 ± 0.6	10	22.7 ± 0.3	101.7	10	22.3 ± 0.5	99.6	10
22	23.1 ± 0.7	10	23.7 ± 0.3	102.6	10	23.1 ± 0.6	100.3	10
29	23.6 ± 0.8	10	24.6 ± 0.5	104.2	10	23.7 ± 0.6	100.2	10
36	25.4 ± 1.1	10	27.2 ± 0.5	107.3	10	24.5 ± 0.8	96.7	10
43	25.2 ± 1.1	10	27.5 ± 0.5	108.9	10	24.6 ± 0.8	97.4	10
50	26.1 ± 1.2	10	28.2 ± 0.5	108.1	10	25.0 ± 0.9	96.0	10
58	27.0 ± 1.2	10	29.1 ± 0.6	107.9	10	25.9 ± 1.0	96.0	10
64	27.9 ± 1.4	10	30.0 ± 0.7	107.4	10	26.8 ± 1.0	95.8	10
71	28.8 ± 1.3	10	30.7 ± 0.6	106.3	10	27.5 ± 1.1	95.3	10
78	29.7 ± 1.3	10	31.9 ± 0.8	107.2	10	29.0 ± 1.0	97.5	10
85	30.3 ± 1.3	10	32.5 ± 0.8	107.3	10	29.6 ± 1.0	97.6	10
88	30.2 ± 1.2	10	32.1 ± 0.8	106.2	10	29.4 ± 1.1	97.4	10

[a]Study day 1 is the day animals were placed on study.
[b]Statistical analysis performed using one-way analysis of variance with post hoc Tukey Honest Significant Difference test for all pairwise comparisons. No statistically significant findings were noted at $p \leq 0.05$.
[c]Weights shown are mean ± standard error.
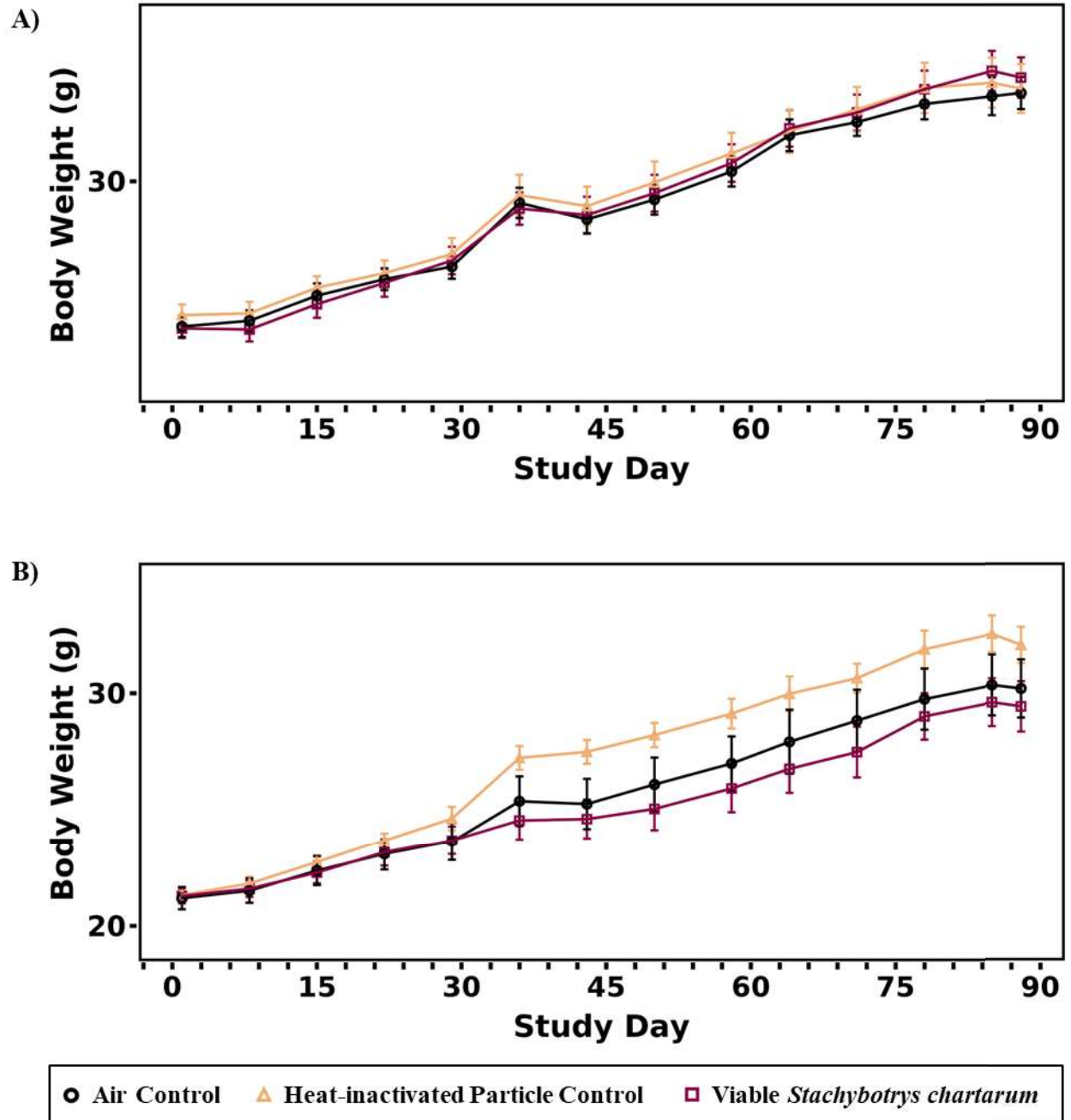
Figure 5. Growth Curves for Male and Female Mice in the Three-month Inhalation Study of *Stachybotrys chartarum*

Growth curves are shown for (A) males and (B) females.

A minimal but significant decrease (<1.3%) in mean cell volume was observed in the viable *S. chartarum*-exposed male mice compared to the air control and heat-inactivated particle control groups (Appendix E). This change was not observed in the viable *S. chartarum*-exposed female mice and was not considered exposure related. Eosinophil counts were significantly increased in the viable *S. chartarum*-exposed male mice relative to the air control and heat-inactivated particle control groups (Table 4). Although not significant, eosinophil counts were higher in the viable *S. chartarum*-exposed female mice relative to the air control and heat-inactivated particle control groups.

Table 4. Summary of Select Hematology Data for Male and Female Mice in the Three-month Inhalation Study of *Stachybotrys chartarum*

Endpoint[a,b]	Air Control	Heat-inactivated Particle Control	Viable *S. chartarum*
Male			
n	9	9[c]	10
Leukocytes (10^3/µL)	3.25 ± 0.29	3.84 ± 0.20	3.77 ± 0.38
Neutrophils (10^3/µL)	0.58 ± 0.04	0.63 ± 0.06	0.48 ± 0.04
Lymphocytes (10^3/µL)	2.52 ± 0.25	3.03 ± 0.16	2.96 ± 0.34
Monocytes (10^3/µL)	0.03 ± 0.01	0.03 ± 0.00	0.08 ± 0.04
Eosinophils (10^3/µL)	0.12 ± 0.01	0.14 ± 0.03	0.25 ± 0.03[**##]
Basophils (10^3/mL)	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Female			
n	6[c]	9[c]	7[c]
Leukocytes (10^3/µL)	2.44 ± 0.40	1.88 ± 0.32	2.57 ± 0.31
Neutrophils (10^3/µL)	0.70 ± 0.11	0.59 ± 0.22	0.67 ± 0.13
Lymphocytes (10^3/µL)	1.66 ± 0.43	1.20 ± 0.20	1.74 ± 0.29
Monocytes (10^3/µL)	0.02 ± 0.01	0.01 ± 0.00	0.05 ± 0.01
Eosinophils (10^3/µL)	0.06 ± 0.01	0.07 ± 0.01	0.11 ± 0.02
Basophils (10^3/mL)	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

[**]Statistically significant pairwise difference from air control at $p \leq 0.01$.
[##]Statistically significant pairwise difference from heat-inactivated particle control at $p \leq 0.01$.
[a]Data are presented as mean ± standard error.
[b]Statistical analysis performed by the Dunn (pairwise) test.
[c]N values differ from number of animals due to insufficient amount of collected sample or coagulation of blood before analysis.

Absolute and relative lung weights were significantly increased in viable *S. chartarum*-exposed female mice compared to the air control group (33% and 35%, respectively) (Table 5). Although not significant, the absolute and relative lung weights of male mice exposed to viable *S. chartarum* were also higher compared to the air control mice (11% and 5%, respectively) (Table 5).

Table 5. Summary of Lung Weights and Lung-Weight-to-Body-Weight Ratios of Male and Female Mice in the Three-month Inhalation Study of *Stachybotrys chartarum*

Endpoint[a,b]	Air Control	Heat-inactivated Particle Control	Viable *S. chartarum*
Male			
n	9	10	10
Necropsy Body Wt. (g)	33.3 ± 0.6	33.4 ± 0.9	33.9 ± 0.8
Lung			
Absolute (g)	0.18 ± 0.01	0.19 ± 0.01	0.20 ± 0.01
Relative (mg/g)[c]	5.50 ± 0.25	5.72 ± 0.17	5.80 ± 0.20
Female			
n	10	10	10
Necropsy Body Wt. (g)	30.2 ± 1.2	32.1 ± 0.8	29.4 ± 1.1
Lung			
Absolute (g)	0.18 ± 0.01	0.17 ± 0.01	0.24 ± 0.01**[##]
Relative (mg/g)	6.07 ± 0.36	5.31 ± 0.29	8.17 ± 0.40**[##]

**Statistically significant pairwise difference from air control at p ≤ 0.01.
[##]Statistically significant pairwise difference from heat-inactivated particle control at p ≤ 0.01.
[a]Data are presented as mean ± standard error.
[b]Statistical analysis performed using one-way analysis of variance with post hoc Tukey Honest Significant Difference test for all pairwise comparisons.
[c]Relative organ weights (organ-weight-to-body-weight ratios) are given as mg organ weight/g body weight.

Histopathology

This section describes the statistically significant or biologically noteworthy changes in the incidences of nonneoplastic lesions of the larynx, lung, and bronchial lymph node.

Gross pathology was evaluated for the organs listed in Table 1. There were no gross lesions identified in any organs evaluated in the male and female groups (Appendix E).

Nonneoplastic lesions in the larynx, lung, and bronchial lymph nodes of viable *S. chartarum*-exposed male and female mice were present in increased numbers compared to air and heat-inactivated particle control groups (Table 6, Table 7, Table 8). The incidences of nonneoplastic lesions were also higher in the larynx of heat-inactivated particle control-exposed female mice compared to the air control.

The presence of fungal material in tissue sections was not observed following hematoxylin and eosin staining. Histological sections of aorta, gallbladder, heart, liver, lung, bronchial lymph node, and spleen were stained with Grocott's methenamine silver (GMS) stain to detect fungal elements. No fungal material was observed in the aorta, gallbladder, heart, liver, or spleen sections.

Larynx: In the larynx of viable *S. chartarum*-exposed male and female mice, the incidence of squamous metaplasia of the epiglottis was significantly increased compared to the air control (males and females) or heat-inactivated particle control (males) groups (Table 6). Squamous metaplasia of the epiglottis was observed in 3/10 female mice in the heat-inactivated particle

control group. The lesions were present at the base of the epiglottis and were characterized by the replacement of normal ciliated cuboidal to columnar epithelium with nonkeratinized squamous epithelium (Figure 6). The metaplasia was graded as minimal (severity score of 1) in all instances and involved <50% of the epithelium overlying the glands at the base of the epiglottis.

Table 6. Incidence of Nonneoplastic Lesions of the Larynx in Male and Female Mice in the Three-month Inhalation Study of *Stachybotrys chartarum*

	Air Control	Heat-inactivated Particle Control	Viable *S. chartarum*
Male			
n[a]	10	10	10
Epiglottis, Metaplasia, Squamous[b]	0	0	4*# (1.0)[c]
Female			
n	10	9	10
Epiglottis, Metaplasia, Squamous	0	3 (1.0)	7** (1.0)

*Statistically significant pairwise difference from air control at $p \leq 0.05$; ** $p \leq 0.01$.
#Statistically significant pairwise difference from heat-inactivated particle control at $p \leq 0.05$.
[a]Number of animals examined microscopically.
[b]Number of animals with lesion. Statistical analysis performed by Fisher's exact (pairwise) one-sided test.
[c]Average severity grade of observed lesion in affected animals: 1 = minimal; 2 = mild; 3 = moderate; 4 = marked.
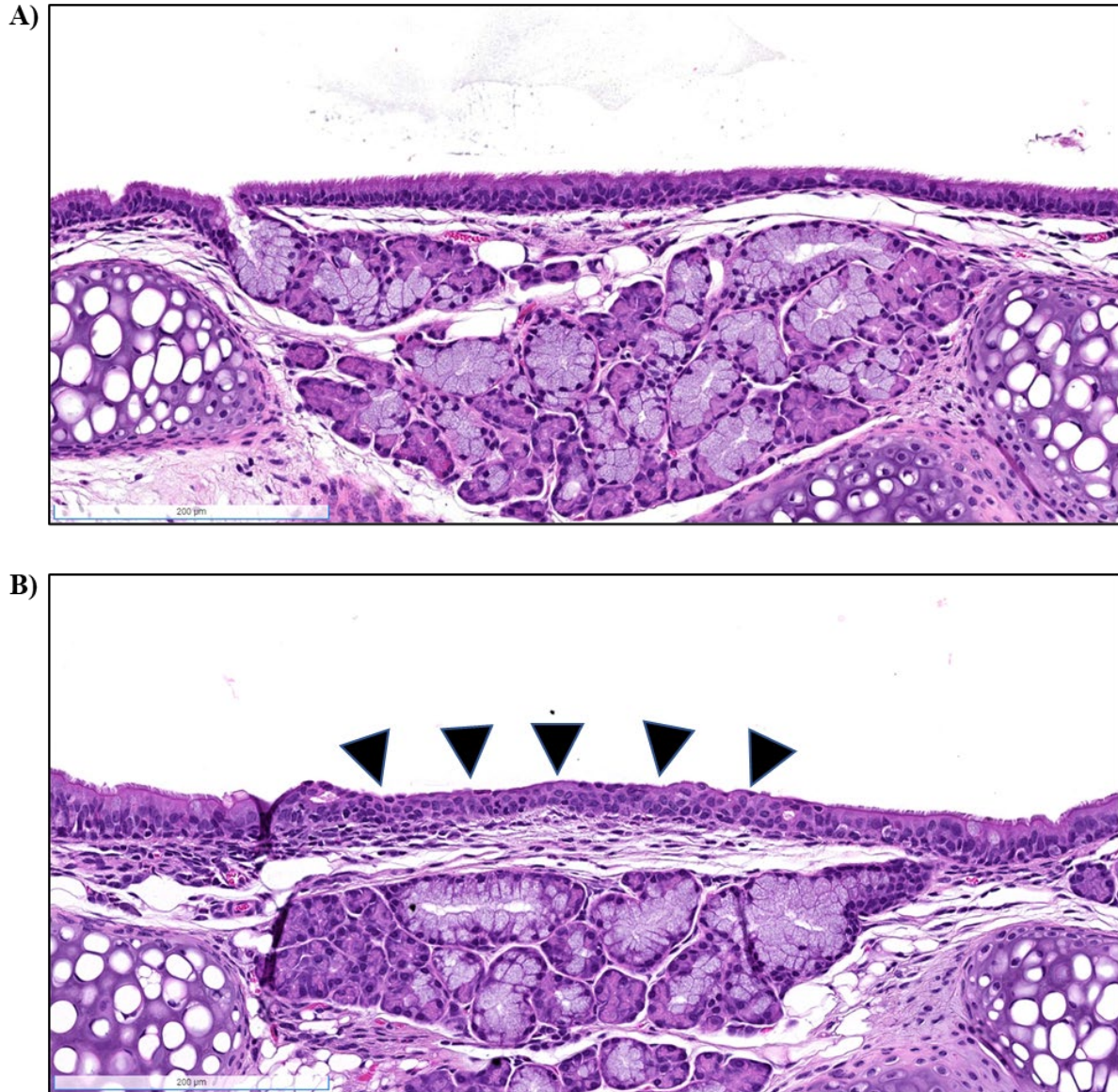
Figure 6. Representative Images of Control Larynx and Squamous Metaplasia in the Larynx of Male and Female Mice in the Three-month Inhalation Study of *Stachybotrys chartarum* (H&E)

Larynx sections from (A) an air control male mouse and (B) a viable *S. chartarum*-exposed female mouse at the base of the epiglottis (40×). In the air control mouse, the epithelium at the base of the epiglottis has normal ciliated columnar epithelium overlying the seromucinous glands. In the viable *S. chartarum*-exposed mouse, minimal squamous metaplasia, indicated by the arrowheads, was seen as replacement of the normal ciliated epithelium by nonkeratinized squamous epithelium.
H&E = hematoxylin and eosin stain.

Lung: Numerous changes were observed in the lungs of viable *S. chartarum*-exposed mice compared to the air control and heat-inactivated particle control groups, which both displayed no lesions. Perivascular chronic-active inflammation was evident in all the viable *S. chartarum*-exposed males and females and was not observed in the air control or heat-inactivated particle control groups (Table 7). Chronic-active inflammation was characterized by the predominantly perivascular accumulations of lymphocytes, neutrophils, eosinophils, and macrophages in

pulmonary artery walls, with extension into the immediately surrounding interstitial tissue (Figure 7).

The perivascular chronic-active inflammation in viable *S. chartarum*-exposed mice was associated with thickening of the small- and medium-sized pulmonary arteries (medial hypertrophy, Figure 7). Mild to moderate medial hypertrophy of pulmonary arteries was present in all male and female viable *S. chartarum*-exposed mice and was not observed in the air control or heat-inactivated particle control groups (Table 6). The medial hypertrophy was characterized by circumferential thickening of the tunica media by concentric layers of hypertrophied and hyperplastic smooth muscle cells of the affected artery walls, accompanied by narrowing of the lumens. In three viable *S. chartarum*-exposed males and one viable *S. chartarum*-exposed female (Table 6), the artery medial hypertrophy was associated with very low numbers of intravascular thrombi (Figure 8). Medial hypertrophy and perivascular chronic-active inflammation severity was graded based upon the proportion of pulmonary arteries affected as follows: minimal severity (1) was <10% affected, mild (2) was 10%–40% affected, moderate (3) was 41%–75% affected, and marked (4) was >75% affected.

Bronchiolar epithelial hyperplasia was observed in all viable *S. chartarum*-exposed male and female mice and was not observed in the air control or heat-inactivated particle control groups (Table 7). Minimal bronchiolar epithelial hyperplasia was characterized by crowded, plump, often piled (2–5 cell layers thick), cuboidal, ciliated epithelial cells lining terminal bronchioles, with extension into adjacent alveolar septa (Figure 7). In most cases, the bronchiolar epithelial hyperplasia involved <10% of bronchioles and was considered minimal (1) but was graded as mild (2) in one male viable *S. chartarum*-exposed mouse in which 10%–20% of the bronchioles were affected.

Lymphocyte hyperplasia of the bronchus-associated lymphoid tissue (BALT) in the lung was observed in 9/10 viable *S. chartarum*-exposed males and females and was not observed in the air control or heat-inactivated particle control groups (Table 7). Lymphocyte hyperplasia of the BALT consisted of an increase in the size and/or number of aggregates of lymphocytes adjacent to bronchi when compared with that observed in air control animals. Air control animals had 0 to 1 lymphoid aggregates (most had 0). The severity of BALT lymphocyte hyperplasia was minimal (1) in all cases.

Histiocytic cellular infiltration in the lung was observed in all viable *S. chartarum*-exposed male and female animals (Table 7). The infiltration was characterized by a minimal to moderate increase in the numbers of diffusely scattered macrophages and rare multinucleated giant cells within alveolar spaces. The lesion severity was graded based upon the proportion of lung affected as follows: minimal severity (1) had <10% of the lung affected, mild (2) had 10%–40% affected, moderate (3) had 41%–75% affected, and marked (4) had >75% affected.

Goblet cell metaplasia was observed in all viable *S. chartarum*-exposed male and female mice and was not observed in the air control or heat-inactivated particle control groups (Table 7). Goblet cell metaplasia affected most to all of the epithelium in an individual airway and was characterized by increased numbers of goblet cells in the epithelium lining the bronchi and/or bronchioles (Figure 7). The severity of this lesion ranged from minimal to moderate and was graded based upon the proportion of airways affected as follows: minimal (1) had <10% of

airways affected, mild (2) had 10%–40% affected, moderate (3) had 41%–75% affected, and marked (4) had >75% affected.

The lung sections of 9/10 male and 10/10 female viable *S. chartarum*-exposed mice stained positive for fungal material (Table 7). In the heat-inactivated particle control groups, GMS-positive fungal material was also observed in 1/10 male mice and 9/10 female mice (Table 7). GMS-positive structures consistent with viable *S. chartarum* conidia were observed in very low numbers (usually <5 throughout the entire lung section) and were round to oval, measuring 3 to 6 μm, and observed intracellularly within alveolar macrophages and occasionally free floating within the alveolar spaces (Figure 9).

Table 7. Incidences of Select Nonneoplastic Lesions of the Lung in Male and Female Mice in the Three-month Inhalation Study of *Stachybotrys chartarum*

	Air Control	Heat-inactivated Particle Control	Viable *S. chartarum*
n[a]	10	10	10
Male			
Perivascular, Inflammation, Chronic-Active[b]	0	0	10**## (2.6)[c]
Artery, Hypertrophy, Medial	0	0	10**## (2.4)
Artery, Thrombus	0	0	3
Bronchiole, Epithelium, Hyperplasia	0	0	10**## (1.1)
BALT, Hyperplasia, Lymphocyte	0	0	9**## (1.0)
Infiltration Cellular, Histiocyte	0	0	10**## (1.8)
Metaplasia, Goblet Cell	0	0	10**## (1.8)
Fungus	0	1	9**##
Female			
Perivascular, Inflammation, Chronic-Active	0	0	10**## (2.7)
Artery, Hypertrophy, Medial	0	0	10**## (2.9)
Artery, Thrombus	0	0	1
Bronchiole, Epithelium, Hyperplasia	0	0	10**## (1.0)
BALT, Hyperplasia, Lymphocyte	0	0	9**## (1.0)
Infiltration Cellular, Histiocyte	0	0	10**## (2.0)
Metaplasia, Goblet Cell	0	0	10**## (2.5)
Fungus	0	9**	10**

**Statistically significant pairwise difference from air control at $p \leq 0.01$.
##Statistically significant pairwise difference from heat-inactivated particle control at $p \leq 0.01$.
BALT = bronchus-associated lymphoid tissue.
[a]Number of animals examined microscopically.
[b]Number of animals with lesion. Statistical analysis performed by Fisher's exact (pairwise) one-sided test.
[c]Average severity grade of observed lesion in affected animals: 1 = minimal; 2 = mild; 3 = moderate; 4 = marked.
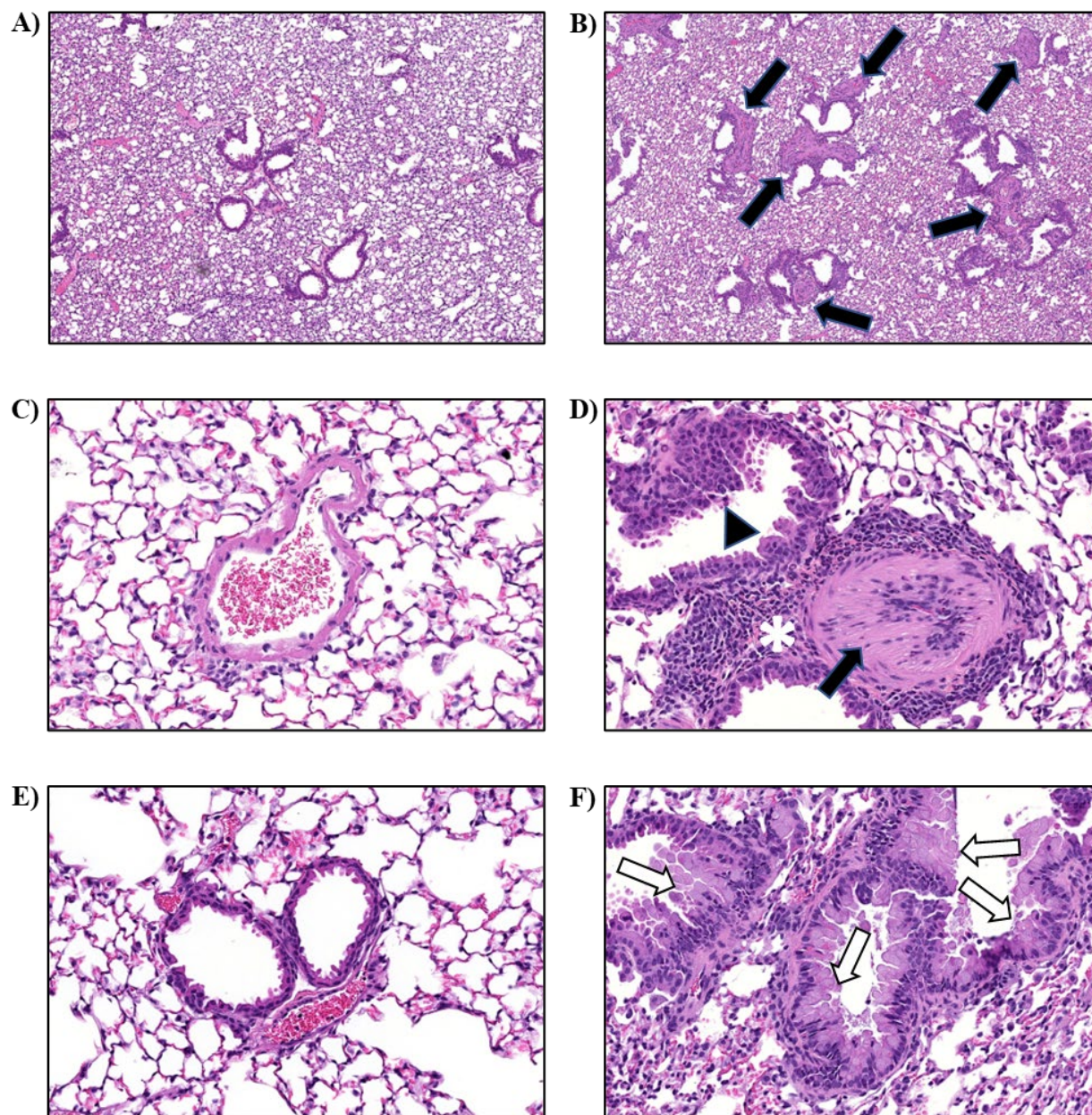
Figure 7. Representative Images of Control Lungs and Perivascular Chronic-Active Inflammation and Medial Hypertrophy of the Pulmonary Artery in the Lungs of Male and Female Mice in the Three-month Inhalation Study of *Stachybotrys chartarum* (H&E)

Lung sections from (A, C, E) air control mice and (B, D, F) viable *S. chartarum*-exposed mice. At low magnification (4×), (B) arteries/arterioles and inflammation (arrows) are prominent in the viable *S. chartarum*-exposed female mouse lung compared to the (A) air control male mouse lung. The (C) normal arteriole from an air control female mouse with thin vessel walls in the lung compared to the (D) arteriole with medial hypertrophy (arrow) in the viable *S. chartarum*-exposed female mouse with circumferential thickening of the tunica media by concentric layers of hypertrophied and hyperplastic smooth muscle cells and narrowing of the vascular lumen (20×). The (D) viable *S. chartarum*-exposed female mouse shows perivascular chronic-active inflammation (white asterisk), seen as accumulations of lymphocytes, neutrophils, and eosinophils and histiocytic cellular infiltrates in surrounding alveoli. The (D) bronchiole in the upper left exhibits bronchiole epithelium hyperplasia (arrowhead), as compared to the (E) bronchiole epithelium in a control male mouse (20×). (F) Goblet cell metaplasia (white arrows) was also seen in bronchioles of viable *S. chartarum*-exposed mice (40×). H&E = hematoxylin and eosin stain.
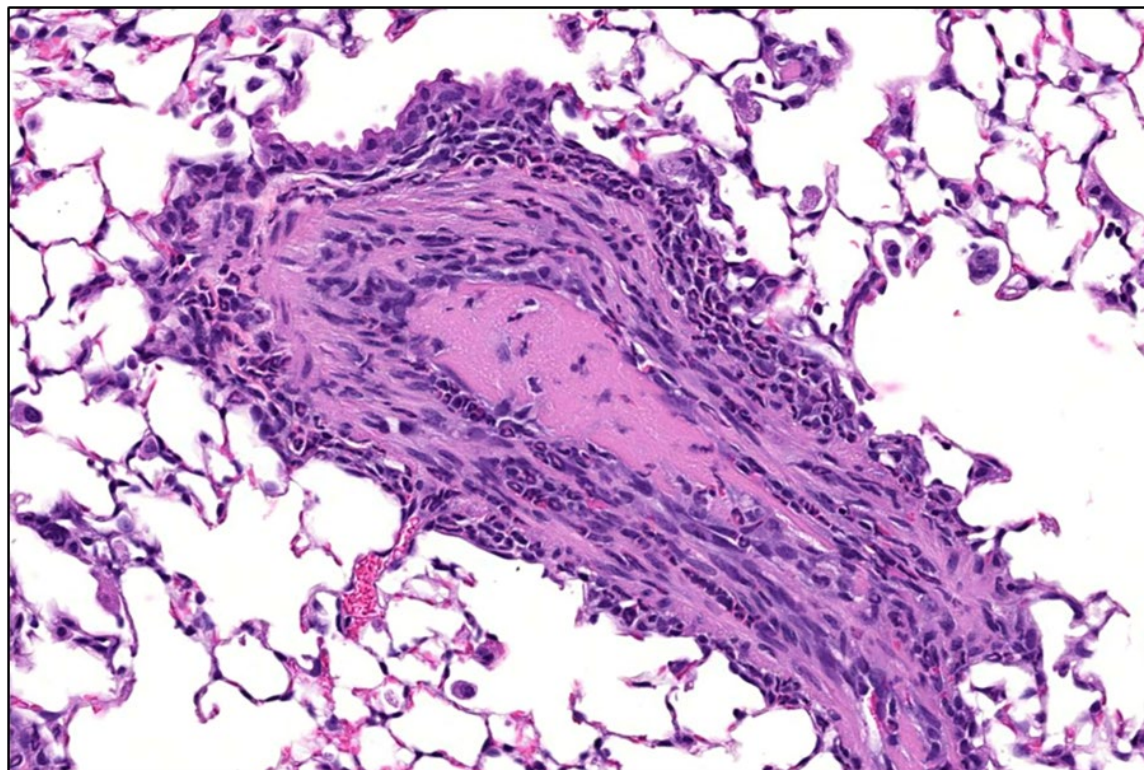
Figure 8. Representative Image of Thrombus in the Lung of a Male Mouse in the Three-month Inhalation Study of *Stachybotrys chartarum* (H&E)

A thrombus in a small artery in the lung from a viable *S. chartarum*-exposed mouse. Thrombi were present in very low numbers within arteries exhibiting medial hypertrophy (40×). Chronic-active perivascular inflammation composed of lymphocytes, neutrophils, and eosinophils within and around the vessel wall and histiocyte infiltration in the surrounding alveolar spaces are also present. H&E = hematoxylin and eosin stain.
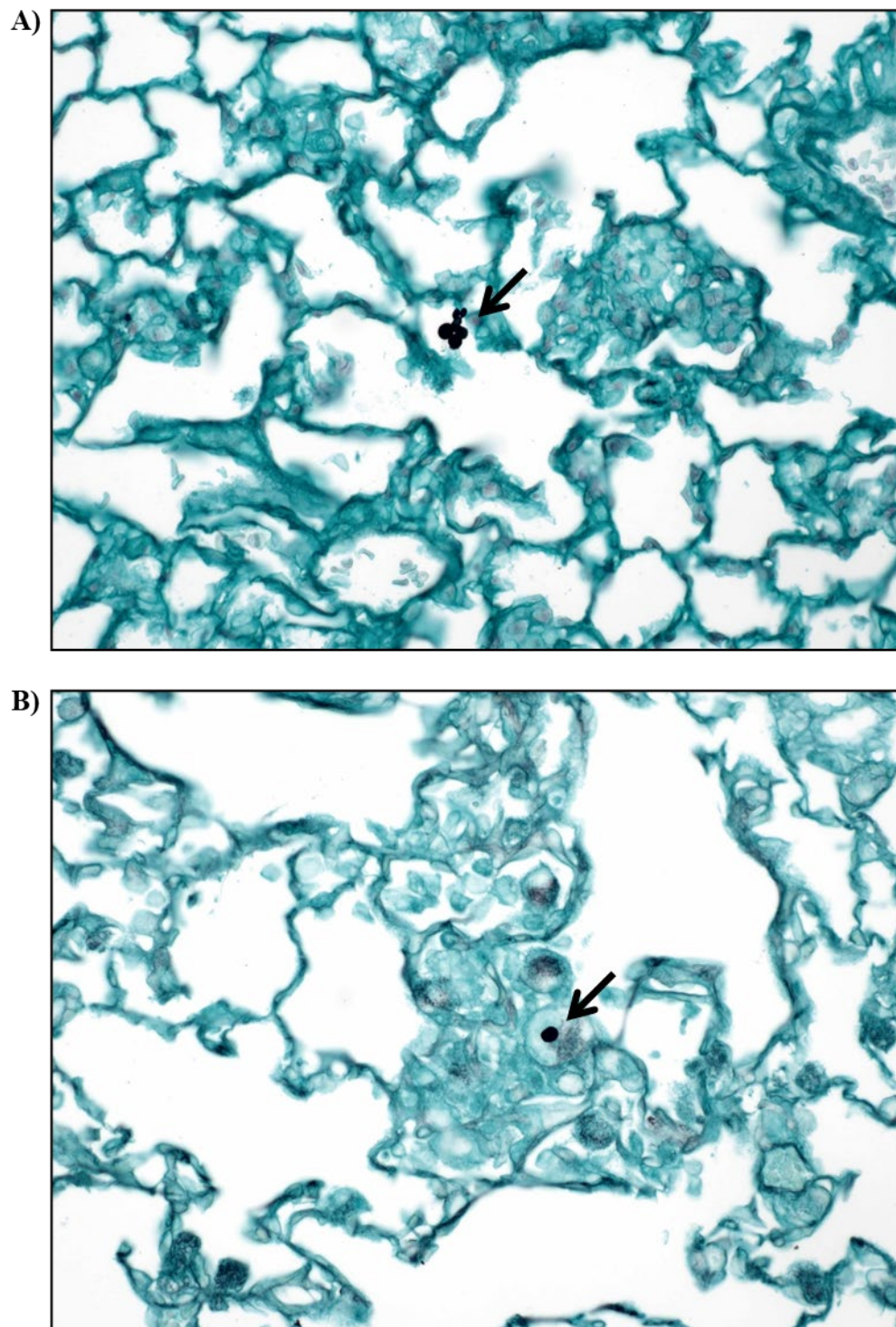
Figure 9. Representative Images of Fungal Conidia Observed within the Lungs of Female Mice in the Three-month Inhalation Study of *Stachybotrys chartarum* (GMS)

Grocott's methenamine silver (GMS)-positive structures consistent with *S. chartarum* conidia were rarely observed, but, when present, were seen free within (A) alveoli or (B) alveolar macrophages. (A: heat-inactivated particle control lung, 40×. B: viable *S. chartarum* lung, 40×).

Bronchial lymph node: Bronchial, mandibular, mediastinal, and mesenteric lymph nodes were analyzed in all study mice. Histopathological analysis showed no visible lesions in the mandibular, mediastinal, or mesenteric lymph nodes. In the bronchial lymph node, lymphocyte hyperplasia was observed in two female viable *S. chartarum*-exposed mice but not in the viable *S. chartarum*-exposed male mice (Table 8). No lesions were observed in the bronchial lymph nodes of air control or heat-inactivated particle control mice. Lymphocyte hyperplasia was minimal in severity and characterized by increased numbers of lymphocytes within the cortex, paracortex, and medullary regions (Figure 10).

Very low numbers of conidia were also observed in the bronchial lymph node of the two female viable *S. chartarum*-exposed mice that also had lymphocyte hyperplasia of the bronchial lymph node (Table 8).

Table 8. Incidences of Select Nonneoplastic Lesions of the Bronchial Lymph Node in Male and Female Mice in the Three-month Inhalation Study of *Stachybotrys chartarum*

	Air Control	**Heat-inactivated Particle Control**	**Viable *S. chartarum***
Male			
n[a]	10	10	8
Hyperplasia, Lymphocyte[b]	0	0	0
Fungus	0	0	0
Female			
n	10	8	8
Hyperplasia, Lymphocyte	0	0	2 (1.0)[c]
Fungus	0	0	2

[a]Number of animals examined microscopically.
[b]Number of animals with lesion. Statistical analysis performed by Fisher's exact (pairwise) one-sided test. No statistically significant findings were noted at $p \leq 0.05$.
[c]Average severity grade of observed lesion in affected animals: 1 = minimal; 2 = mild; 3 = moderate; 4 = marked.
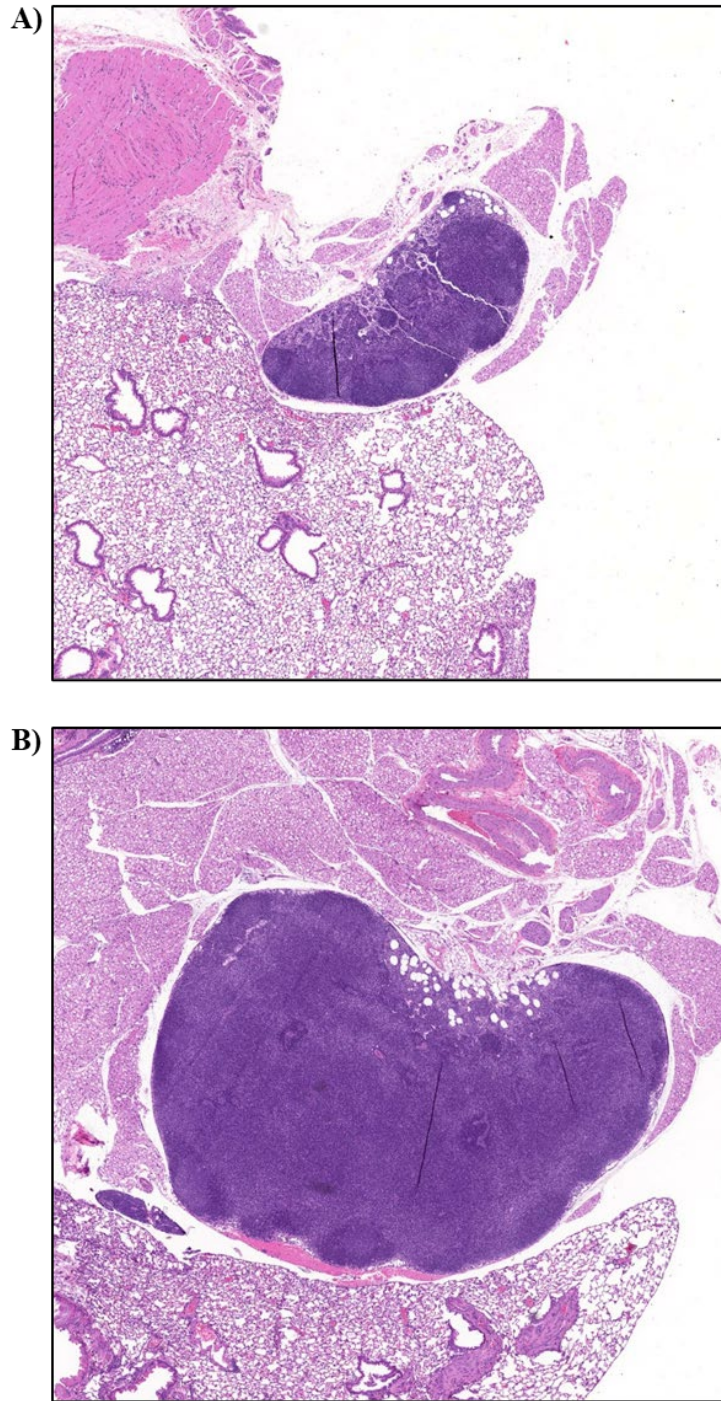
Figure 10. Representative Images of Control Lungs with Bronchial Lymph Node and Lymphocyte Hyperplasia in the Bronchial Lymph Nodes of Female Mice in the Three-month Inhalation Study of *Stachybotrys chartarum* (H&E)

At the same magnification, the bronchial lymph node from (A) an air control mouse is smaller than the bronchial lymph node from (B) a viable *S. chartarum*-exposed mouse with lymphocyte hyperplasia, which was seen as increased numbers of lymphocytes in multiple compartments, including the cortex, follicles, paracortex, and medullary cords (2×). H&E = hematoxylin and eosin stain.

Genetic Toxicology

No significant increases in the frequencies of micronucleated reticulocytes and mature erythrocytes were observed in the peripheral blood of male or female B6C3F1/N mice exposed to either viable *S. chartarum* or heat-inactivated particle control conidia relative to the air control mice (Table D-1). Similarly, no significant increase in frequency was observed in mice exposed to viable *S. chartarum* conidia compared to mice exposed to the heat-inactivated particle control. In addition, no significant alteration in the percentage of reticulocytes was observed in these mice, suggesting no effects on hematopoiesis. These negative results in the micronucleus test indicate an absence of exposure-induced chromosomal damage in progenitor erythrocytes in the bone marrow of mice, along with an absence of generalized bone marrow toxicity. Data from all NTP genetic toxicity tests with *S. chartarum* are available in the NTP CEBS database: https://doi.org/10.22427/NTP-DATA-TOX-107.[121]

Discussion

Stachybotrys chartarum is a hydrophilic fungus that requires a cellulose-rich substrate and a high moisture content for optimal growth.[35-38] This species is often identified in water-damaged indoor environments and has a worldwide distribution.[35-38; 122] Following infant acute idiopathic pulmonary hemorrhage cases,[14] as well as the association with "sick building syndrome" and damp building-related illness,[44; 46; 59; 62-66] the adverse health effects following *S. chartarum* exposure have become a concern for public health. *S. chartarum* phialides produce conidia (spores) within a slime droplet that protects the conidia over a long period and inhibits aerosolization. Once dried, the conidia, hyphae, and fragments may be aerosolized within the breathing zone following abiotic or biotic disturbances. While most *S. chartarum* conidia are too large to deposit deep within the murine lung, this is not the case with the small respirable-size fungal fragments present within this test article.

Although mycotoxicosis and pulmonary immunological endpoints have been previously examined,[29; 101] the systemic toxicity following subchronic inhalation of viable *S. chartarum* remains undercharacterized. To evaluate the toxicological responses to *S. chartarum*, groups of 10 male and 10 female B6C3F1/N mice inhaled viable *S. chartarum* conidia, heat-inactivated *S. chartarum* conidia (biological particle control), or high-efficiency particulate air [filter] (HEPA)-filtered air (control) twice a week for 3 months. An estimated pulmonary dose of 1×10^4 *S. chartarum* conidia (viable and heat-inactivated conidia) was chosen for this study, which was the highest aerosolized dose that reproducibly induced a lymphoproliferative response and was the same concentration and schedule used for a previously published pulmonary immunology study.[27] This estimated lung burden would be equivalent to that of a worker breathing a constant workplace air concentration of 3.45×10^6 conidia/m^3 (146 µg/m^3) for 1 week, working 8 hours per day for 5 days or equivalent to a worker breathing 2.3×10^4 conidia/m^3 (0.97 µg/m^3) for 8 hours per day, 5 days per week over a 40-year working career. This concentration is comparable to the occupational exposure concentration of 10^3–10^7 conidia/m^3, which has been reported to result in toxicosis.[43] In addition, studies involving *S. chartarum*-contaminated residences have also reported spore concentrations around 4×10^3 conidia/m^3 during remediation[86] and 10^3 conidia/mg of dust collected from contaminated areas.[123]

In the current study, inhalation exposure to an estimated pulmonary dose of 1×10^4 viable *S. chartarum* conidia twice a week for 3 months did not elicit overt toxicity or result in >10% body weight loss compared to the air control group for male or female mice. Additionally, the terminal body weights of mice exposed to either viable *S. chartarum* or the heat-inactivated particle control were not significantly different from those of the air control group. Although not significant, the absolute and relative lung weights of male mice exposed to viable *S. chartarum* were slightly higher compared to the air control group, whereas the viable *S. chartarum*-exposed female mice had significantly increased absolute and relative lung weights compared to the air control group.

No gross lesions were present following exposure; however, the incidences of nonneoplastic lesions were significantly increased in mice exposed to viable *S. chartarum*. These histopathological changes were evident in the lung and, to a lesser extent, in the larynx and bronchial lymph nodes of viable *S. chartarum*-exposed mice. Within the rest of the respiratory

tract, no histopathological changes were observed in the nose or trachea. In the larynx of both male and female viable *S. chartarum*-exposed mice, the incidence of squamous metaplasia at the base of the epiglottis was significantly increased compared to the air control mice. In addition, squamous metaplasia at the base of the epiglottis was also observed in the larynx of three female mice exposed to the heat-inactivated particle control. The squamous metaplasia of the larynx epithelium at the base of the epiglottis included replacement of ciliated cuboidal to columnar cells with nonkeratinized stratified squamous epithelium, which is in response to chronic irritation elicited from the viable *S. chartarum* exposure. The National Toxicology Program (NTP) study detailing the toxicological responses following a subchronic *Aspergillus fumigatus* exposure also reported similar laryngeal squamous metaplasia at the base of the epiglottis in mice exposed to viable *A. fumigatus*.[30] Literature has suggested that the larynx is the site that often responds to repeated inhalation of substances, including fungi, and that the resultant squamous metaplasia is frequently of minimal severity.[30; 124] Additionally, in the bronchial lymph node, lymphocyte hyperplasia was observed in two viable *S. chartarum*-exposed female mice, but no lesions were observed in *S. chartarum*-exposed male mice or in the bronchial lymph nodes of air control or heat-inactivated particle control mice.

Histopathological analysis of the lungs of viable *S. chartarum*-exposed males and females showed nonneoplastic lesions, including perivascular chronic-active inflammation, histiocytic cellular infiltration, medial hypertrophy of pulmonary arteries, thrombi in arteries with medial hypertrophy, hyperplasia of bronchiolar epithelium, goblet cell metaplasia, and bronchus-associated lymphoid tissue (BALT) lymphocyte hyperplasia. The mild to moderate medial hypertrophy affected small- and medium-sized pulmonary arteries and was characterized by circumferential thickening of the tunica media and luminal narrowing and was accompanied by accumulations of inflammatory cells. Intravascular thrombi, which were present in three males and one female exposed to *S chartarum*, were observed in very low numbers of arterioles that were remodeled as a result of medial hypertrophy. Despite the low incidence and number of affected vessels, the thrombi were considered likely exposure related because they were associated with vascular changes of luminal narrowing and medial hypertrophy. Similar to the current study, previously reported *S. chartarum* exposure via inhalation (twice per week for 4 weeks or 3 months) in female B6C3F1/N mice resulted in peribronchiolar and perivascular inflammation, alveolar histiocytosis, bronchiolar epithelial cell hyperplasia, as well as pulmonary arterial remodeling characterized by the thickening of the pulmonary arterial wall and narrowing of the lumen.[27] Neither the air control nor heat-inactivated particle control groups in this study, as well as the previously published pulmonary immunology exposure study,[27] presented evidence of medial hypertrophy of arteries. In addition, separate studies showed the development of pulmonary arterial hypertension in mice following repeated intratracheal instillation of *S. chartarum*,[25; 125] accompanied by significantly increased right ventricular systolic pressure and right ventricular hypertrophy in exposed male ddY mice compared to the control mice.[25] Although cardiac function and pathology were not evaluated in the current study, the heart weights of the *S. chartarum*-exposed mice were not significantly different compared to the air control group, which is in contrast to the study conducted by Ochiai et al.[25] These divergent results could be explained by the difference in delivery methods or the difference in mouse or fungal strain. Interestingly, cessation of *S. chartarum* exposure via intratracheal instillation resulted in the resolution of the pulmonary remodeling[125]; however, further investigation is warranted to determine whether this resolution occurs following repeated inhalation of viable *S. chartarum* conidia.

In comparison to the current study, an NTP study conducted in collaboration with the National Institute for Occupational Safety and Health (NIOSH) describing the toxicological responses following a subchronic viable *A. fumigatus* exposure showed similar pulmonary arterial medial hypertrophy.[30] In addition, chronic-active inflammation, defined as the increased presence of neutrophils and perivascular infiltrates, including alveolar macrophages and eosinophils, was observed in mice exposed to viable *A. fumigatus*,[30] and similar cell populations were observed following subchronic viable *S. chartarum* exposure. The significantly increased eosinophils observed in male mice may correspond to the chronic-active inflammation in the lung following subchronic viable *S. chartarum* exposure.

Perivascular chronic-active inflammation observed in the current study was characterized by an influx of inflammatory cell populations, including lymphocytes, neutrophils, eosinophils, and macrophages in the pulmonary arterial walls with extension into the immediately adjacent interstitial tissue and alveoli. In addition, histiocytic cellular infiltrates were observed in all viable *S. chartarum*-exposed male and female mice. All findings were also observed in female B6C3F1/N mice in a previously published study, following a 3-month exposure of viable *S. chartarum* via inhalation.[27] Croston and colleagues showed, in the analysis of the bronchoalveolar lavage fluid (BALF), a significant increase in neutrophils, macrophages, and eosinophils at 24 hours and 48 hours post final exposure following a 3-month exposure to *S. chartarum*.[27] In agreement, Nagayoshi et al. showed a significant increase in eosinophils accompanied by high concentrations of T helper 2 cell (Th2)-associated cytokines, interleukin (IL)-4 and IL-5, in the BALF following a 12-week intratracheal instillation of *S. chartarum* in male ddY mice.[125] Using male BALB/c mice, Rosenblum Lichtenstein and colleagues also showed significantly increased BALF neutrophils, lymphocytes, and eosinophils and significantly increased IL-4, IL-5, and IL-6, supporting a Th2 response following a 7-week *S. chartarum* intratracheal instillation.[126] Using the same mouse strain, Leino et al. reported significantly increased inflammatory cells including monocytes, neutrophils, and lymphocytes in response to intranasal instillation (twice a week for 3 weeks) of 1×10^5 satratoxin-producing and nonsatratoxin-producing *S. chartarum* spores in female mice.[127] No inflammatory cellular infiltrates were observed following exposure to 1×10^3 satratoxin-producing and nonsatratoxin-producing *S. chartarum* spores.[127] Additionally, proinflammatory cytokines were induced in the lungs of *S. chartarum*-exposed mice,[127] and in contrast to previously published data,[125; 126] there was no significant increase in the expression of hallmark Th1 or Th2 cytokines. Although cytokine expression was not measured in the current study, Croston and colleagues showed that a 4-week exposure to viable *S. chartarum* resulted in a Th2-mediated response that switched to a mixed T-cell response after 3 months of exposure, as evidenced by significantly increased *ifng* (interferon-gamma; IFN-γ), *il2* (IL-2), and *il13* (IL-13) gene expression in the lungs of exposed mice.[27] This mixed T-cell response is in contrast to other literature reporting a Th2-mediated response following 7 weeks[126] and 12 weeks[125] of *S. chartarum* exposure and, as previously stated, could be due to the difference in exposure methods or the different mouse and fungal strains used for the studies. While literature describes the evidence of Th2-mediated pulmonary arterial remodeling after 12 weeks of *S. chartarum* exposure,[25; 27; 125] this pathological observation has been observed as early as 4 weeks following exposure to the same exposure schedule and strain of *S. chartarum* used in the current study.[27] Ochiai et al.[25] reported the development of pulmonary arterial thickening in *S. chartarum*-exposed mice after 4 weeks of intratracheal instillation. Previous studies suggest that the duration of *S. chartarum* exposures,[27;

[126] the route of delivery,[23-26; 128] fungal strain,[27] and the animal model employed[129] could influence the resulting immune responses and lung pathologies.

Studies have indicated that different strains of mice respond differently following *S. chartarum* exposure.[129] Specifically, BALB/c (Th2-biased) mice showed a different lung injury profile and inflammatory-associated biochemical and cellular profile than C57BL/6J (Th1-biased)[126; 129] and C3H/HeJ mice.[129] The hybrid B6C3F1/N mouse strain used in the current study has been widely used to study systemic toxicological responses and was selected for consistency with other NTP toxicology studies. This strain is resistant to disease and has low spontaneous disease rates compared with other strains and hybrids.[130] Although the female parent of the hybrid B6C3F1/N mouse strain, C57BL/6, is Th1-biased, histopathological results of the airways are consistent with previously observed histopathology in other strains of mice, including ddY mice.[25] Moreover, the ddY mouse strain has been previously used as a model of *S. chartarum*-induced idiopathic pulmonary arterial hypertension.[131] For the studies using the ddY mouse model, the *S. chartarum* test article was administered via intratracheal instillation.[25; 125; 131] Compared to intratracheal and intranasal instillation or liquid aerosol inhalation that deliver conidia in a liquid suspension,[23-26; 128] which can potentially modify the viability and components of the conidia,[42] the current study used a previously developed system to deliver dry aerosolized conidia to mice housed in nose-only exposure chambers, simulating a natural exposure that could be experienced in a *S. chartarum*-contaminated environment. Although the *S. chartarum* test article in the current study was not grown on building materials as has been previously used, the use of rice as a nutrient-rich growth substrate eliminates the potential interference of building material exposure to the mice housed in the exposure units. These differences in the administration of the *S. chartarum* conidia could partly account for the contrasting results observed among the different exposure studies. In addition, the strain of *S. chartarum* used in the study may also be a parameter that could influence the pulmonary responses following repeated exposure. As previously reported,[27] a higher-fragmenting strain of *S. chartarum* (IBT 9460) elicited an earlier pulmonary immune response and pulmonary arterial remodeling compared to a lesser fragmenting strain of *S. chartarum* (IBT 7711), indicating that smaller fragments may deposit deeper in the lungs and induce these responses. Therefore, the higher-fragmenting *S. chartarum* strain (IBT 9460) was selected for toxicological evaluation in the current study. Furthermore, the cultivation and aerosolization of IBT 9460 has been previously optimized.[28] In the study conducted by Lemons et al., both the viable *S. chartarum* test article (IBT 9460) and the heat-inactivated particle control contained similar levels of verrucarol, a hydrolysis product of macrocyclic trichothecene, which were four times higher than the *S. chartarum* (IBT 7711) strain. In addition, the endotoxin level of the viable *S. chartarum* test article was near or below the limit of detection.[28]

In the current 3-month study, very low numbers of conidia were observed free in the alveoli or within alveolar macrophages of most viable *S. chartarum*-exposed mice (Figure 9) and in the bronchial lymph node of two viable *S. chartarum*-exposed female mice. Following a 4-week intratracheal instillation of *S. chartarum* spores in male ddY mice, spores were shown to be present for only 24 hours before being cleared by alveolar macrophages.[125] In addition, fungal components were not visualized in left lung lobes following a 3-month inhalation exposure to *S. chartarum* in female B6C3F1 mice,[27] which is contrary to the histopathological observations of the current study. Similar to the current study, *A. fumigatus* spores were detected within the lungs of mice following exposure; however, in a few cases, swollen conidia and germ tubes were

detected, indicating active germination of the conidia,[30; 100; 102] which was not observed in the current study.

Hyphal fragments can also be aerosolized within an indoor environment following abiotic or biotic disturbance. Studies have demonstrated that fungal fragments contribute to the fungal biomass within indoor environments.[10; 11] Studies of *S. chartarum* have predicted that fragments can be aerosolized at particle concentrations 500 times higher than spores.[8] Furthermore, deposition of submicron fungal fragments deep within the human respiratory tract is 230–250 fold higher than that of spores, which would primarily be deposited in the upper respiratory tract.[8] Croston and colleagues have recently proposed fragments as the primary driver of pulmonary immune responses following the inhalation of *S. chartarum*.[27] Moreover, literature has suggested that the macrocyclic trichothecenes detected on *S. chartarum* fragments may contribute to the toxigenic health effects following exposure.[37; 47] In addition to macrocyclic trichothecenes, other biologically active products of *S. chartarum*, such as hemolysins, proteinases, glucans, and volatile organic compounds, could act in concert to contribute to the toxigenic health effects following exposure.[37] A study conducted by Hudson et al. demonstrated pulmonary immune response differences in the magnitude and temporal interval following intratracheal instillation exposure to conidia of macrocyclic-trichothecene-producing (JS 58-17) and nonproducing chemotype (JS 58-06) *S. chartarum*.[132] Additionally, Rand and colleagues determined that pulmonary tissue collected from CFW male mice following a single intratracheal instillation of *S. chartarum* spores responds differently compared to pure trichothecene exposure (isosatratoxin F), including significantly decreased alveolar air space and significantly increased erythrocytes in the intra-alveolar air space.[133] Although mycotoxin profiles and concentrations were not evaluated in the current study, Lemons et al. showed similar levels of verrucarol in the viable and heat-inactivated particle control aerosols of the same strain of *S. chartarum*.[28] Furthermore, Croston et al. showed that a more rapid immune response and pulmonary remodeling occurred following exposure to this higher-fragmenting and mycotoxin-producing strain of *S. chartarum* (IBT 9460).[27] As the peer-reviewed literature has suggested, fragment size, mycotoxin content, or a combination of these factors could be responsible for the immune response and arterial remodeling observed; therefore, further research is warranted.[134]

Findings from the current 3-month inhalation study in B6C3F1/N mice included significantly increased absolute and relative lung weights in viable *S. chartarum*-exposed female mice, relative to the heat-inactivated control and air control groups accompanied by significantly increased incidences of nonneoplastic lesions. In males and females exposed to viable *S. chartarum*, nonneoplastic lesions were restricted mainly to the larynx and lung; however, there was also a low incidence of lymphocyte hyperplasia in the bronchial lymph nodes of viable *S. chartarum*-exposed female mice. In the larynx, nonneoplastic lesions consisted of squamous metaplasia of the epithelium localized at the base of the epiglottis. Lung histopathology findings were composed of several lesions, including medial hypertrophy of the pulmonary arteries/arterioles in viable *S. chartarum*-exposed mice but not in heat-inactivated particle control mice. These results correspond with the results from a previously published subchronic *S. chartarum* pulmonary immunology study conducted by NIOSH with the same exposure system that showed Th2-mediated pulmonary inflammation and pulmonary arterial remodeling after subchronic *S. chartarum* inhalation exposure.[27] The results of the current study were also similar to the previously published NTP report detailing the toxicological responses to *A. fumigatus* exposure in which the incidence of nonneoplastic lesions was significantly

increased in the larynx, lung, and bronchial lymph nodes of viable *A. fumigatus*-exposed mice.[30] Specifically, the incidences of bronchial lymphocytic hyperplasia and plasma cell hyperplasia were significantly increased in viable *A. fumigatus*-exposed mice but not in viable *S. chartarum*-exposed mice, relative to either the air control or the heat-inactivated particle control groups.[30] In both the current study of *S. chartarum* and the previously published NTP report of *A. fumigatus*, lungs of mice exposed to viable conidia displayed inflammation, medial hypertrophy of pulmonary arterioles and arteries, bronchiolar epithelium hyperplasia, goblet cell metaplasia, BALT lymphocyte hyperplasia, and the presence of very low numbers of conidia.

Under the conditions of this 3-month study, target organs identified in B6C3F1/N mice following inhalation exposure to viable *S. chartarum* conidia were the larynx, lung, and (to a lesser extent) the bronchial lymph nodes. The histopathological changes observed following viable *S. chartarum* exposure were not seen in the heat-inactivated particle control or air control mice. These results build on initial pulmonary immunology studies conducted at NIOSH using the same exposure parameters and demonstrate that the immunological responses and histopathology could be mediated by the viable *S. chartarum* bioaerosol.

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Appendix A. Culture, Characterization, and Delivery of *Stachybotrys chartarum*

Table of Contents

Tables

A.1. Reconstitution, Culture, and Glycerol Stock Preparation of *Stachybotrys chartarum*

The *Stachybotrys chartarum* strain (CBS 414.95, IBT 9460) used in this study was originally isolated from a water-damaged building in Finland. Malt extract agar (MEA) culture plates were aseptically inoculated with 20 µL of the stored glycerol stock in a class II biological safety cabinet (BSC). MEA culture plates were incubated at 26°C for 10–14 days for optimal spore growth. *S. chartarum* conidia (spores) were harvested by liberating the conidia in 2 mL of sterile water using an inoculating loop. The conidia suspension was mixed with an equal volume of 50% glycerol, and 0.5-mL aliquots of the suspension were prepared in cryogenic storage vials. Glycerol stock preparations were stored at −80°C for the duration of the study.

A.2. Preparation of the Viable *Stachybotrys chartarum* Test Article

One aliquoted frozen glycerol conidia stock was thawed, and two MEA culture plates were aseptically inoculated with 20 µL of the stock suspension in a BSC. Plates were incubated at 26°C for 12–14 days. *S. chartarum* conidia were harvested from each plate by liberating the conidia in 2 mL of sterile water (W3500, Sigma Aldrich, St. Louis, MO) using an inoculating loop. The concentration of the conidia suspension was determined by diluting the suspension 1:100, and conidia counts were determined using a hemocytometer. The conidia suspension was diluted to 2.5×10^5 conidia/mL using sterile water. Mahatma-enriched extra-long grain white rice (30 g) and distilled deionized water (10 mL) were added to twelve 250-mL flasks covered with aluminum foil, autoclaved at 121°C for 30 minutes, and allowed to cool in a BSC. Once cooled, these 12 flasks were aseptically inoculated with 5 mL of the 2.5×10^5 conidia/mL suspension, recapped with foil, and incubated at 26°C for 21–28 days. The flasks were shaken vigorously one time after 1 week of growth to prevent aggregation of the rice grains. At the end of the growth phase, the foil caps were punctured using aseptic technique and the flasks were laid horizontally in a biological safety cabinet to increase the surface area during the drying phase. Cultures were placed in a desiccator for 7–10 days for drying prior to aerosolization. Preparation of the viable test article for the first week of exposures was initiated 1 month before the start of the 3-month exposure period.

A.3. Preparation of *Stachybotrys chartarum* Heat-inactivated Particle Control

After 21–28 days of culture, 6 of the 12 fungi-coated rice culture flasks were immersed in a dry bead bath (Lab Armor, Irving, TX) set to 80°C for 2 hours and allowed to cool. As with the viable test articles, the heat-inactivated particle control flasks were then placed in a desiccator for 7–10 days before aerosolization.

A.4. Determination of the Reduction in *Stachybotrys chartarum* Conidia Viability in the Heat-inactivated Particle Control

S. chartarum conidia were harvested from heat-inactivated particle control cultures every other week by removing 10–20 random conidia-laden rice grains from the cultures and placing them in a 15-mL tube containing 5 mL sterile water. Conidia suspensions were diluted to

1×10^5 conidia/mL, and 100 µL were plated on MEA culture plates (1×10^4 conidia/plate). After 48–96 hours of growth at 25°C, fungal colony-forming units (CFU) were counted to determine the number of viable *S. chartarum* conidia/mL. The percentage of viable conidia in the heat-inactivated particle control was then calculated. The cultures were categorized as nonviable if >95% of the conidia were not viable.

A.5. Assessment of Fungal Contaminants in the Exposure Articles

Genomic DNA was extracted from the heat-inactivated particle control and viable *S. chartarum* cultures every other week to analyze fungal contaminants using the Roche High Pure PCR Template Preparation Kit (Roche, Indianapolis, IN). Conidia were harvested from 10–20 conidia-laden rice grains in sterile distilled, deionized water. Conidia from 200 µL of the suspension were pelleted by centrifugation and suspended in Roche Tissue Lysis Buffer (200 µL) containing 5 µL of CelLytic B Cell Lysis Reagent (Sigma Aldrich, St. Louis, MO); tubes were incubated at 37°C for 30 minutes. Next, 200 µL Roche Binding Buffer and 40 µL proteinase K solution was added, and tubes were incubated at 70°C for 10 minutes. After the addition of 100 µL of isopropanol, extracted DNA was washed and eluted according to the manufacturer's recommendations. Internal transcribed spacer (ITS) regions of fungal ribosomal RNA were amplified using the Fun18Sf/ITS4R primer pair, and purified amplicons were cloned into a pDRIVE vector that was then transformed into chemically competent *Escherichia coli*. *E. coli* clones positive for the ITS amplicon insert were used to inoculate 96-well plates for the generation of glycerol stocks. The glycerol stocks were sequenced with the T7 and SP6 primers using Sanger sequencing. Forward and reverse sequences were trimmed and assembled and then clustered into operational taxonomic units (groups of sequences with ≥97% similarity). Representative sequences from each operational taxonomic unit were searched against sequences deposited in the National Center for Biotechnology Information database and identified based on sequence identity. Any sequences identified as a fungal species other than *S. chartarum* or *Stachybotrys* spp. were considered fungal contaminants. The number of sequences analyzed was dependent on the number of viable conidia in the sample. Very low DNA yield from heat-inactivated particle control samples was a result of heat treatment. The number of contaminant fungal sequences is shown relative to the number of sequences analyzed in each sample in Table A-1.

A.6. Aerosolization of the Exposure Articles Using the Acoustical Generator System

Prior to aerosolization of the exposure test articles, the viable *S. chartarum* and heat-inactivated particle control cultures were placed in a desiccator for 7–10 days to remove excess moisture and allow optimal aerosolization. Fungal-laden rice was placed on a rubber membrane housed on top of a speaker within the acoustical generator system (AGS), a modified Pitt-3 generator[95] linked to a multianimal nose-only exposure chamber. The Pitt-3 generator was purchased from the University of Pittsburgh and evaluated under conditions recommended by its engineering school.[96] The exposure chamber consisted of a vertical cylindrical tower with 24 ports (4 levels of 6 ports) holding animal pods that projected outward radially (Figure 2). The system was fitted with a computer controller, further modified to its current design,[97; 98] and used by the National Institute for Occupational Safety and Health (NIOSH) to study the respiratory effects caused by

the inhalation of a wide variety of occupationally relevant dusts. The AGS was initially used as a new and improved system for dispersing nanomaterials[99] and was subsequently modified for fungal spore aerosolization as described by Buskirk et al.[100] Two AGS systems were used in this study: one for the viable *S. chartarum* exposures and one for the heat-inactivated particle control exposures. Air control mice were placed in an identical exposure chamber that was not attached to the AGS but received the same high-efficiency particulate air [filter] (HEPA)-filtered air delivered to the test article chamber. Twenty animal pods were attached to each exposure chamber, 10 for male mice and 10 for female mice. Animals were randomly placed in the pods for each exposure to minimize the effects of potential minor variations in conidia concentrations at each port. The additional ports served as sample ports to collect mass concentration, particle size, and samples on filters for field emission electron microscopy analysis (Hitachi S-4800, Tokyo, Japan).

The fungal-laden rice was allowed to settle within the AGS before the start of exposure. Acoustical energy was then used to aerosolize fungal conidia, which were delivered into the exposure chamber at a flow rate of 6 L/min. Particle size and mass concentration data were needed to estimate the total lung particle deposition during each exposure. Three pods were reserved as sample ports. The first port was used to gravimetrically measure the mass concentration, and the second used a light-scattering device (DataRAM4, ThermoElectron Co., Franklin, MA) to provide real-time estimates of the mass concentration (*Con*) during exposures. The gravimetric filter data were used to validate and calibrate the DataRAM4 every exposure. The third sample pod was used to collect aerodynamic particle size data (APS, TSI Inc., Soreview, MN). The APS data were collected the week before the start of animal exposures. To collect these data, the system was run in an identical manner as an animal exposure without animals present, and when the real-time mass concentration reading was stable, several 2-minute APS samples were taken. The average DataRam4 readings during the 2-minute APS samples were noted (*Con_DuringAPS*).

An estimate of the total number of particles deposited in the upper and lower airways was calculated each second (*TPDs*) by the custom Labview software during each exposure run, using the following equation:

$$TPDs = S \ x \ Con$$

Wherein:

S = deposition scale factor, particle number/(mg/m^3)

Con = real-time DataRam4 concentration, mg/m^3

The deposition scale factor (*S*), used in the above equation, was calculated before the beginning of animal exposures with the following equation:

$$S = \frac{MV \ x \ \sum_{i=1}^{i=52}\{Ncon(i) \ x \ \left(DF\_Alveolar(i) + DF\_Tracheo(i)\right)\}}{(Con\_DuringAPS \ x \ 60 \ sec.)}$$

Wherein:

MV = estimated animal breathing minute volume, set to 25 for the current study, mL

i = midpoint of each of the 52 APS aerodynamic size classification channels, ranging from 0.5 to 20 μm

Ncon(i) = the number concentration of particles that fall within the smallest bin size range

DF_Alveolar(i) = mouse lung alveolar deposition fraction for particle size *i*, ranging from 0 to 1[b]

DF_Tracheo(i) = mouse lung tracheobronchial deposition fraction for particle size *i*, ranging from 0 to 1[b]

Con_DuringAPS = average mass concentration during the APS measurement, mg/m^3

The custom Labview software would keep a running total of particles deposited, and when the estimate reached the target daily dose, the particle generator would shut off and deliver HEPA-filtered air to the exposure chamber for the remainder of the 60-minute exposure interval. If the estimated particles deposited did not reach the target within 1 hour, the exposure was stopped, and the estimated count of particles deposited was noted. For the current study, the estimated pulmonary deposition was 1×10^4 conidia, equating to approximately 7 mg/m^3 heat-inactivated conidia and 8 mg/m^3 viable fungal conidia.

Table A-2 and Table A-3 describe the measured exposure concentrations of each exposure. APS data are shown in Table A-4. The columns represent the three different methods of producing size distribution curves (as seen in Figure 3). The aerodynamic particle sizer totals the particles in each particle size by number, by the surface area of the particles in each size bin, and by the mass of the particles in each size bin. Field emission electron microscopy analysis showed that the aerosol primarily consisted of single conidia within the 3–5 μm range (Figure 3).

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[b]The mouse-lung-deposition model was based on interpolated data from Raabe et al.[103]

Table A-1. Weekly Evaluation of Viable *Stachybotrys chartarum* and Heat-inactivated Particle Control

	Heat-inactivated Particle Control	Viable *S. chartarum*
Viability[a]		
Viable Spore Concentration	1.37×10^3 conidia/mL ($\pm 1.62 \times 10^3$)	ND
Percentage Nonviable	98.63% (±1.62%)	–
Contamination[b]		
Week 1	0/0[c]	0/76
Week 3	0/40	0/42
Week 5	0/39	0/93
Week 7	0/0[c]	0/0[c]
Week 9	0/43	0/92
Week 11	0/0[c]	0/85
Week 13	0/0[c]	0/88

ND = not determined.
[a]Data are presented as mean (± standard deviation).
[b]DNA derived from *S. chartarum* was the only fungal DNA detected in heat-inactivated particle control and test article cultures. Heat inactivation destroyed the DNA in heat-inactivated particle controls most weeks and resulted in fewer or no fungal sequences to analyze compared to viable test articles.
[c]Limited genomic DNA yield did not allow for completion of sequence analysis.

Table A-2. Weekly Viable *Stachybotrys chartarum* Exposure Concentrations

Exposure Day	Chamber Concentration (DataRAM [mg/m³])	Chamber Concentration (Filter [mg/m³])	Filter Weight (mg)	Number of Conidia Deposited in Lung
1	8.16	8.34	0.501	1.011×10^4
3	8.13	7.32	0.440	1.009×10^4
8	7.96	6.01	0.370	1.010×10^4
10	8.01	7.85	0.480	1.009×10^4
15	8.10	8.54	0.512	1.008×10^4
17	7.97	7.56	0.454	1.009×10^4
22	8.06	8.10	0.490	1.008×10^4
24	8.03	8.20	0.500	1.008×10^4
30	7.66	8.28	0.530	1.013×10^4
32	8.02	10.82	0.660	1.009×10^4
36	8.12	8.49	0.510	1.008×10^4
38	8.16	8.53	0.510	1.009×10^4
43	7.84	8.74	0.540	1.011×10^4
45	7.94	8.02	0.497	1.010×10^4
50	8.11	9.09	0.545	1.007×10^4

Exposure Day	Chamber Concentration (DataRAM [mg/m³])	Chamber Concentration (Filter [mg/m³])	Filter Weight (mg)	Number of Conidia Deposited in Lung
52	8.12	10.57	0.630	1.009×10^4
58	7.62	7.58	0.485	1.008×10^4
60	8.00	10.02	0.611	1.013×10^4
64	7.77	9.44	0.595	1.005×10^4
66	8.10	6.87	0.412	1.009×10^4
71	7.97	9.21	0.562	1.008×10^4
73	8.04	8.58	0.523	1.007×10^4
78	7.50	6.90	0.449	1.009×10^4
80	7.91	9.56	0.593	1.007×10^4
85	8.10	9.34	0.560	1.008×10^4
87	8.01	8.97	0.547	1.009×10^4
Mean[a]	7.98 ± 0.17	8.50 ± 1.12	0.520 ± 0.067	$1.009 \times 10^4 \pm 17.51$

[a]Data are presented as mean ± standard deviation of the measurement over the course of the study.

Table A-3. Weekly Heat-inactivated Particle Control Exposure Concentrations

Exposure Day	Chamber Concentration (DataRAM [mg/m³])	Chamber Concentration (Filter [mg/m³])	Filter Weight (mg)	Number of Conidia Deposited in Lung
1	7.08	6.56	0.390	1.019×10^4
3	7.10	7.54	0.450	1.023×10^4
8	7.06	9.81	0.590	1.016×10^4
10	7.05	8.06	0.480	1.020×10^4
15	7.13	8.66	0.515	1.028×10^4
17	7.07	6.40	0.384	1.032×10^4
22	7.14	9.25	0.560	1.029×10^4
24	7.15	8.70	0.520	1.027×10^4
30	7.14	9.37	0.570	1.029×10^4
32	7.08	8.83	0.530	1.017×10^4
36	6.80	9.59	0.590	1.011×10^4
38	6.99	9.55	0.570	1.008×10^4
43	7.27	9.48	0.570	1.049×10^4
45	6.96	9.77	0.596	1.019×10^4
50	7.04	4.89	0.293	1.014×10^4
52	7.07	6.02	0.360	1.018×10^4
58	6.94	5.78	0.353	1.021×10^4
60	7.08	5.62	0.337	1.026×10^4

Exposure Day	Chamber Concentration (DataRAM [mg/m³])	Chamber Concentration (Filter [mg/m³])	Filter Weight (mg)	Number of Conidia Deposited in Lung
64	7.12	7.39	0.444	1.028×10^4
66	7.10	7.10	NA	1.026×10^4
71	7.15	9.07	0.544	1.029×10^4
73	7.11	8.26	0.496	1.023×10^4
78	6.79	6.04	0.374	1.017×10^4
80	7.08	8.64	0.518	1.018×10^4
85	7.03	8.06	0.491	1.029×10^4
87	6.99	9.41	0.574	1.028×10^4
Mean[a]	7.06 ± 0.10	7.99 ± 1.50	0.484 ± 0.092	$1.023 \times 10^4 \pm 81.84$

NA = not available.
[a]Data are presented as mean ± standard deviation of the measurement over the course of the study.

Table A-4. Aerodynamic Particle Size Statistics of Aerosolized *Stachybotrys chartarum* Based on Aerodynamic Particle Sizer Measurements

	Number (Particle Size)	Surface (Particle Size)	Mass (Particle Size)
Viable *S. chartarum*			
Median (µm)	2.88 ± 0.12	4.26 ± 0.03	4.43 ± 0.03
Mean (µm)	2.75 ± 0.05	4.14 ± 0.03	4.43 ± 0.03
Geometric Mean (µm)	2.22 ± 0.05	3.95 ± 0.03	4.30 ± 0.02
Mode (µm)	4.27 ± 0.17	4.37 ± 0.00	4.37 ± 0.00
Geometric Standard Deviation	2.02 ± 0.01	1.41 ± 0.01	1.29 ± 0.01
Heat-inactivated Particle Control			
Median (µm)	3.77 ± 0.17	4.10 ± 0.11	4.26 ± 0.11
Mean (µm)	3.77 ± 0.22	4.21 ± 0.16	4.44 ± 0.20
Geometric Mean (µm)	3.63 ± 0.26	4.10 ± 0.16	4.32 ± 0.17
Mode (µm)	3.84 ± 0.11	4.02 ± 0.11	4.12 ± 0.12
Geometric Standard Deviation	1.34 ± 0.07	1.25 ± 0.03	1.25 ± 0.03

Values represent the average ± standard deviation of aerodynamic particle sizer measurements taken in 2-minute durations for the test article (n = 3) and heat-inactivated particle control (n = 6).

Appendix B. Ingredients, Nutrient Composition, and Contaminant Levels in NTP-2000 Mouse Ration

Tables

Table B-1. Ingredients of NTP-2000 Mouse Ration

Ingredients	Percent by Weight
Ground Hard Winter Wheat	23.00
Ground #2 Yellow Shelled Corn	22.44
Wheat Middlings	15.0
Oat Hulls	8.5
Alfalfa Meal (Dehydrated, 17% Protein)	7.5
Purified Cellulose	5.5
Soybean Meal (49% Protein)	4.0
Fish Meal (60% Protein)	4.0
Corn Oil (without Preservatives)	3.0
Soy Oil (without Preservatives)	3.0
Dried Brewer's Yeast	1.0
Calcium Carbonate (USP)	0.9
Vitamin Premix[a]	0.5
Mineral Premix[b]	0.5
Calcium Phosphate, Dibasic (USP)	0.4
Sodium Chloride	0.3
Choline Chloride (70% Choline)	0.26
Methionine	0.2

USP = United States Pharmacopeia.
[a]Wheat middlings as carrier.
[b]Calcium carbonate as carrier.

Table B-2. Vitamins and Minerals in NTP-2000 Mouse Ration

	Amount[a]	Source
Vitamins		
Vitamin A	4,000 IU	Stabilized vitamin A palmitate or acetate
Vitamin D	1,000 IU	D-activated animal sterol
Vitamin K	1.0 mg	Menadione sodium bisulfite complex
α-Tocopheryl Acetate	100 IU	–
Niacin	23 mg	–
Folic Acid	1.1 mg	–
α-Pantothenic Acid	10 mg	α-Calcium pantothenate
Riboflavin	3.3 mg	–
Thiamine	4 mg	Thiamine mononitrate
B_{12}	52 µg	–
Pyridoxine	6.3 mg	Pyridoxine hydrochloride

	Amount[a]	Source
Biotin	0.2 mg	d-Biotin
Minerals		
Magnesium	514 mg	Magnesium oxide
Iron	35 mg	Iron sulfate
Zinc	12 mg	Zinc oxide
Manganese	10 mg	Manganese oxide
Copper	2.0 mg	Copper sulfate
Iodine	0.2 mg	Calcium iodate
Chromium	0.2 mg	Chromium acetate

[a]Per kg of finished diet.

Table B-3. Nutrient Composition of NTP-2000 Mouse Ration

Nutrient	Mean ± Standard Deviation	Range	Number of Samples
Protein (% by Weight)	14.3	–	1
Crude Fat (% by Weight)	7.9	–	1
Crude Fiber (% by Weight)	9.49	–	1
Ash (% by Weight)	5.05	–	1
Amino Acids (% of Total Diet)			
Arginine	0.808 ± 0.073	0.67–0.97	31
Cystine	0.220 ± 0.021	0.15–0.25	31
Glycine	0.703 ± 0.037	0.62–0.8	31
Histidine	0.341 ± 0.068	0.27–0.68	31
Isoleucine	0.548 ± 0.039	0.43–0.66	31
Leucine	1.096 ± 0.061	0.96–1.24	31
Lysine	0.070 ± 0.101	0.31–0.86	31
Methionine	0.409 ± 0.040	0.26–0.49	31
Phenylalanine	0.623 ± 0.045	0.471–0.72	31
Threonine	0.513 ± 0.040	0.43–0.61	31
Tryptophan	0.156 ± 0.026	0.11–0.2	31
Tyrosine	0.425 ± 0.064	0.28–0.54	31
Valine	0.666 ± 0.038	0.55–0.73	31
Essential Fatty Acids (% of Total Diet)			
Linoleic	3.936 ± 0.229	3.49–4.55	31
Linolenic	0.304 ± 0.030	0.21–0.368	31

Nutrient	Mean ± Standard Deviation	Range	Number of Samples
Vitamins			
Vitamin A (IU/kg)	3,270	–	1
α-Tocopherol (ppm)	2,376 ± 12,602	13.6–69,100	30
Thiamine (ppm)[b]	7.4	–	1
Riboflavin (ppm)	8.32 ± 2.868	4.2–17.5	31
Niacin (ppm)	79.78 ± 8.978	66.4–98.2	31
Pantothenic Acid (ppm)	26.28 ± 10.69	17.4–81.0	31
Pyridoxine (ppm)[b]	9.832 ± 2.080	6.44–14.3	31
Folic Acid (ppm)	1.61 ± 0.434	1.15–3.27	31
Biotin (ppm)	0.319 ± 0.113	0.0–0.704	31
B_{12} (ppb)	48.82 ± 33.79	18.3–174.0	31
Choline (as Chloride) (ppm)	2,553 ± 632	1,160–3,790	31
Minerals			
Calcium (%)	0.943	–	1
Phosphorus (%)	0.573	–	1
Potassium (%)	0.663 ± 0.035	0.563–0.733	31
Chloride (%)	0.389 ± 0.044	0.3–0.517	31
Sodium (%)	0.194 ± 0.027	0.153–0.283	31
Magnesium (%)	0.216 ± 0.052	0.185–0.49	31
Iron (ppm)	190.0 ± 35.69	135–311	31
Manganese (ppm)	49.87 ± 9.15	21.0–73.1	31
Zinc (ppm)	56.53 ± 24.87	42.5–184	31
Copper (ppm)	7.64 ± 2.42	3.21–16.3	31
Iodine (ppm)	0.50 ± 0.232	0–0.972	31
Chromium (ppm)	1.164 ± 1.16	0.33–3.97	30
Cobalt (ppm)	0.217 ± 0.148	0.086–0.864	29

[a]From formulation.
[b]As hydrochloride.

Table B-4. Contaminant Levels in NTP-2000 Mouse Ration

	Mean	Number of Samples
Contaminants		
Arsenic (ppm)	0.252	1
Cadmium (ppm)	0.051	1
Lead (ppm)	0.066	1
Mercury (ppm)	0.01	1
Selenium (ppm)	0.145	1
Aflatoxins (ppb)[a]	<5.0	1
Nitrate Nitrogen (ppm)[b]	11.5	1
Nitrite Nitrogen (ppm)[a,b]	<0.12	1
BHA (ppm)[a,c]	<1.0	1
BHT (ppm)[a,c]	<1.0	1
Aerobic Plate Count (CFU/g)[a]	<10.0	1
Coliform (MPN/g)[a]	<3	1
Escherichia coli (MPN/g)[a]	<10	1
Salmonella sp. (MPN/g)	Negative	1
Total Nitrosamines (ppb)[d]	9.1	1
N-Nitrosodimethylamine (ppb)[d]	5.0	1
N-Nitrosopyrrolidine (ppb)[d]	4.1	1
Pesticides (ppm)		
α-BHC[a]	<0.01	1
β-BHC[a]	<0.02	1
γ-BHC[a]	<0.01	1
δ-BHC[a]	<0.01	1
Heptachlor[a]	<0.01	1
Aldrin[a]	<0.01	1
Heptachlor Epoxide[a]	<0.01	1
DDE[a]	<0.01	1
DDD[a]	<0.01	1
DDT[a]	<0.01	1
HCB[a]	<0.01	1
Mirex[a]	<0.01	1
Methoxychlor[a]	<0.05	1
Dieldrin[a]	<0.01	1
Endrin[a]	<0.01	1
Telodrin[a]	<0.01	1
Chlordane[a]	<0.05	1
Toxaphene[a]	<0.01	1
Estimated PCBs[a]	<0.20	1

	Mean	Number of Samples
Ronnel[a]	<0.01	1
Ethion[a]	<0.02	1
Trithion[a]	<0.05	1
Diazinon[a]	<0.10	1
Methyl Chlorpyrifos	0.19	1
Ethyl Chlorpyrifos	<0.025	1
Methyl Pirimiphos	<0.02	1
Methyl Parathion[a]	<0.025	1
Ethyl Parathion[a]	<0.02	1
Malathion[a]	<0.02	1
Endosulfan I[a]	<0.02	1
Endosulfan II	0.12	1
Endosulfane Sulfate[a]	<0.01	1

All samples were irradiated.
BHA = butylated hydroxyanisole; BHT = butylated hydroxytoluene; CFU = colony-forming units; MPN = most probable number; BHC = hexachlorocyclohexane or benzene hexachloride; DDE = dichlorodiphenyldichloroethylene; DDD = dichlorodiphenyldichloroethane; DDT = dichlorodiphenyltrichloroethane; HCB = hexachlorobenzene; PCB = polychlorinated biphenyl.
[a]All values were below the detection limit. The detection limit is given as the mean.
[b]Sources of contamination include alfalfa, grains, and fish meal.
[c]Sources of contamination include soy oil and fish meal.
[d]All values were corrected for percent recovery.

Appendix C. National Institute for Occupational Safety and Health Sentinel Animal Program

Table of Contents

Tables

C.1. Methods

The sentinel animal program at the National Institute for Occupational Safety and Health (NIOSH) monitors for common rodent pathogens in each study room to ensure study mice are healthy and pathogen free. Pathogens are monitored through the collection of blood and feces from sentinel mice housed in the study rooms.

For this toxicity study, mice weighing >10% or <10% of the mean weight of all male or female study mice were selected for the sentinel group (n = 20; 10/sex). Females were group housed with five per cage. Male mice were single housed. For each week the mice were on study, sentinel animals were exposed to dirty bedding from study animals. The 4-week sentinels were evaluated for viral antibodies, *Helicobacter*, and pinworms 24 hours following the second exposure in the fourth exposure week. The 3-month sentinels were evaluated only at study termination. All sentinels were euthanized at study termination and evaluated for abnormalities by gross necropsy.

Whole blood was collected via tail vein bleed for 4-week sentinels or via cardiac puncture postmortem for 3-month sentinels. Three to four drops of whole blood were placed onto an Opti-Spot card (IDEXX Laboratories, Inc., Westbrook, ME) for serology tests. These cards were submitted to IDEXX BioResearch for analysis using the Opti-HM serology profile. The profile included *Mycoplasma pulmonis*, ectromelia virus, epizootic diarrhea of infant mice virus, lymphocytic choriomeningitis virus, mouse hepatitis virus, minute virus of mice, mouse parvovirus, mouse norovirus, pneumonia virus of mice, reovirus type 3, Sendai virus, and Theiler's murine encephalomyelitis virus.

One fecal pellet from each sentinel mouse was collected with sterile forceps, and all pellets for each sample group were placed in a single sterile sample tube for analysis (maximum of 10 pellets/tube). Samples were submitted to IDEXX BioResearch for polymerase chain reaction (PCR) analysis using the *Helicobacter* profile. The profile included *Helicobacter bili*, *H. ganmani*, *H. hepaticus*, *H. mastomyrinus*, *H. rodentium*, *H. typhlonius*, and *Helicobacter* spp. Fur pelt swabs were also collected from each 4-week sentinel mouse and submitted to IDEXX BioResearch for PCR evaluation of the pinworms *Aspiculuris tetraptera* and *Syphacia obvelata*. Swabs were pooled for the analysis.

C.2. Results

All test results were negative (Table C-1).

Table C-1. Methods and Results for Sentinel Animal Testing in Male and Female Mice

	Three-month Study	
Collection Time Points	**4 Weeks**	**Study Termination**
Number Examined (Males/Females)	5/5	5/5
Method/Test		
Multiplex Fluorescent Immunoassay (MFI)[a]		
Ectromelia virus	–	–
Epizootic diarrhea of infant mice (EDIM)	–	–
Lymphocytic choriomeningitis virus	–	–
Mycoplasma pulmonis	–	–
Mouse hepatitis virus (MHV)	–	–
Mouse norovirus (MNV)	–	–
Mouse parvovirus (MPV)	–	–
Minute virus of mice (MVM)	–	–
Pneumonia virus of mice (PVM)	–	–
Reovirus type 3 (REO3)	–	–
Sendai	–	–
Theiler's murine encephalomyelitis virus (TMEV) GDVII	–	–
PCR Evaluation		
Helicobacter[b]	–	–
Pinworms[c]	–	NT

– = negative; PCR = polymerase chain reaction; NT = not tested.
[a]Serology profiles were conducted on whole blood samples of individual sentinel mice (n = 5/group).
[b]PCR evaluations for *Helicobacter* species were conducted on pooled fecal samples for each group.
[c]PCR evaluations for pinworms were conducted on pooled fur pelt swabs.

Appendix D. Genetic Toxicology

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Tables

D.1. Evaluation Protocol

National Toxicology Program (NTP) reports consider biological as well as statistical factors to determine an overall assay result. For an individual assay, the statistical procedures for data analysis are described in the following protocols. There have been instances, however, in which multiple samples of a chemical were tested in the same assay, and different results were obtained among these samples and/or among laboratories. In such cases, all the data are critically evaluated with attention given to possible protocol variations in determining the weight of evidence for an overall conclusion of chemical activity in an assay. For in vitro assays conducted with and without exogenous metabolic activation, results obtained in the absence of activation are analyzed separately from results obtained in the presence of activation. The summary table in the abstract of this Toxicity Report presents the scientific judgment of the Division of Translational Toxicology (DTT) and the National Institute for Occupational Safety and Health (NIOSH) regarding the overall evidence for activity of the chemical in an assay.

D.2. Micronucleus Assay

D.2.1. Peripheral Blood Micronucleus Test Protocol

Peripheral blood samples were analyzed by Integrated Laboratory Systems, LLC (ILS; Research Triangle Park, NC) for determination of erythrocyte micronucleus frequencies. Briefly, ethylenediaminetetraacetic acid (EDTA)-stabilized peripheral blood samples were shipped on ice packs immediately following terminal necropsy from NIOSH to ILS. Before ILS received the samples, microcentrifuge tubes containing anticoagulant (heparin) were prepared and stored at 4°C, and 15 mL conical tubes containing fixative (methanol) were prepared and stored at $-80°C \pm 5°C$.

Upon arrival, blood samples were fixed in ultracold methanol using a MicroFlowPLUS Kit (Litron Laboratories, Rochester, NY) according to the manufacturer's instructions. Fixed samples were stored in a $-80°C$ freezer until analysis. Thawed blood samples were analyzed for frequency of micronucleated immature erythrocytes (polychromatic erythrocytes or PCEs, reticulocytes) and mature erythrocytes (normochromatic erythrocytes or NCEs) using a flow cytometer[135]; both the mature and immature erythrocyte populations can be analyzed separately by employing special cell surface markers to differentiate the two cell types. In mice, both the mature and immature erythrocyte populations can be evaluated for micronucleus frequency because the mouse spleen does not sequester and eliminate damaged erythrocytes. Damaged erythrocytes achieve steady state in the peripheral blood of mice following 4 weeks of continuous exposure. Approximately 20,000 reticulocytes and 1×10^6 erythrocytes were analyzed per animal for the frequency of micronucleated cells, and the percentage of immature erythrocytes (% PCE) was calculated as a measure of bone marrow toxicity resulting from test article exposure.

For most NTP studies, the statistical methods for micronucleus assays employ a trend test followed by Dunn pairwise tests. Due to the design of this study (with one exposed group and two control groups), no trend test is needed, and the Dunn test was used for the three-way pairwise comparison of the control and exposed groups. The Dunn test has a built-in correction

for multiple comparisons so the overall false positive rate of the experiment can be maintained. Pairwise comparisons with the control groups are considered statistically significant at $p \leq 0.05$.

Historical control data are used to evaluate the biological importance of any observed response. Both statistical significance and biological importance are considered when arriving at a call. The results of statistical analyses, reproducibility of any effects observed, and the magnitudes of those effects are all considered when determining the final call.

D.2.2. Results

No significant increases in the frequencies of micronucleated reticulocytes and mature erythrocytes were observed in the peripheral blood of male or female B6C3F1/N mice exposed to either viable *S. chartarum* or heat-inactivated particle control conidia relative to the air control mice (Table D-1). Similarly, no significant increase in frequency was observed in mice exposed to viable *S. chartarum* conidia compared to mice exposed to the heat-inactivated particle control. In addition, no significant alteration in the percentage of reticulocytes was observed in these mice, suggesting no effects on hematopoiesis. These negative results in the micronucleus test indicate an absence of exposure-induced chromosomal damage in progenitor erythrocytes in the bone marrow of mice, along with an absence of generalized bone marrow toxicity.

Table D-1. Frequency of Micronuclei in Peripheral Blood Erythrocytes of Male and Female Mice in the Three-month Inhalation Study of *Stachybotrys chartarum*

	Number of Mice with Erythrocytes Scored	MN-RET/1,000[a]	P Value[b]	MN-ME/1,000[a]	P Value[b]	% RET[a]	P Value[b]
Male							
Air Control	5	3.120 ± 0.12		1.636 ± 0.04		1.844 ± 0.06	
Heat-inactivated Particle Control	5	3.200 ± 0.37	1.000	1.672 ± 0.05	1.000	1.769 ± 0.09	0.688
Viable *S. chartarum*[c]	5	3.040 ± 0.12	1.000/1.000	1.618 ± 0.08	1.000/0.688	1.764 ± 0.08	1.000/1.000
Female							
Air Control	5	1.820 ± 0.15		1.027 ± 0.03		1.900 ± 0.10	
Heat-inactivated Particle Control	5	2.120 ± 0.18	0.384	1.084 ± 0.04	0.867	2.031 ± 0.07	1.000
Viable *S. chartarum*[c]	5	1.960 ± 0.11	1.000/1.000	1.004 ± 0.05	1.000/0.609	1.954 ± 0.21	1.000/1.000

MN-RET = micronucleated reticulocytes; MN-ME = micronucleated mature erythrocytes; RET = reticulocytes.
[a]Data are presented as mean ± standard error.
[b]Pairwise comparisons with the air or heat-inactivated particle control groups were performed by the Dunn test.
[c]P values for the viable *S. chartarum* groups are presented as: p value (viable *S. chartarum* compared to air control)/p value (viable *S. chartarum* compared to heat-inactivated particle control).

Appendix E. Supplemental Data

Tables with supplemental data can be found here: https://doi.org/10.22427/NTP-DATA-TOX-107.[121]

E.1. Three-month *Stachybotrys chartarum* Study Tables - Mice

E.1.1. Data Tables

I01 - Animal Removal Summary
I01_-_Animal_Removal_Summary.pdf

I02 - Animal Removals
I02_-_Animal_Removals.pdf

I03 - Growth Curve
I03_-_Growth_Curve.pdf

I03C - Growth Curve
I03C_-_Growth_Curve.pdf

I04 - Mean Body Weight Summary
I04_-_Mean_Body_Weight_Summary.pdf

I04G - Mean Body Weight Gain Summary
I04G_-_Mean_Body_Weight_Gain.pdf

I05 - Clinical Observations Summary
I05_-_Clinical_Observations_Summary.pdf

PA02 - Neoplastic Lesion Summary with Percent Incidence
PA02_-_Neoplastic_Lesion_Summary_with_Percent_Incidence.pdf

PA03 - Nonneoplastic Lesion Summary with Percent Incidence
PA03_-_Nonneoplastic_Lesion_Summary_with_Percent_Incidence.pdf

PA05 - Incidence Rates of Neoplastic Lesions with Systemic Lesions Abridged
PA05_-_Incidence_Rates_of_Neoplastic_Lesions_with_Systemic_Lesions_Abridged.pdf

PA06 - Organ Weights Summary
PA06_-_Organ_Weights_Summary.pdf

PA10X - Statistical Analysis of Nonneoplastic Lesions
PA10X_-_Statistical_Analysis_of_Nonneoplastic_Lesions.pdf

PA14 - Individual Animal Pathology Data
PA14_-_Individual_Animal_Pathology_Data.pdf

PA18 - Incidence Rates of Nonneoplastic Lesions by Anatomic Site with Average Severity Grade
PA18_-_Incidence_Rates_of_Nonneoplastic_Lesions_by_Anatomic_Site_with_Average_Severity_Grade.pdf

PA43 - Hematology Summary
PA43_-_Hematology_Summary.pdf

PA46 - Summary of Gross Pathology
PA46_-_Summary_of_Gross_Pathology.pdf

E.1.2. Individual Animal Data

Individual Animal Body Weight Data
Individual_Animal_Body_Weight_Data.xlsx

Individual Animal Clinical Observations Data
Individual_Animal_Clinical_Observations_Data.xlsx

Individual Animal Gross Pathology Data
Individual_Animal_Gross_Pathology_Data.xlsx

Individual Animal Hematology Data
Individual_Animal_Hematology_Data.xlsx

Individual Animal Histopathology Data
Individual_Animal_Histopathology_Data.xlsx

Individual Animal Organ Weight Data
Individual_Animal_Organ_Weight_Data.xlsx

Individual Animal Removal Reasons Data
Individual_Animal_Removal_Reasons_Data.xlsx

E.2. Genetic Toxicology

E.2.1. *Stachybotrys chartarum* (67892-26-6) in Micronucleus Study G04052 in Male B6C3F1/N Mice

G04 - In Vivo Micronucleus Summary Data
G04_-_In_Vivo_Micronucleus_Summary_Data.pdf

Individual Animal In Vivo Micronucleus Data
G04_-_Individual_Animal_In_Vivo_Micronucleus_Data.xlsx