Title

Toxins of the death cap mushroom, Amanita phalloides

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Publication Date

2020

Peer reviewed|Thesis/dissertation

Toxins of the death cap mushroom, *Amanita phalloides*

By

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A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Microbiology

in the

Graduate Division

of the

University of California, Berkeley

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Spring 2020

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Abstract

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Unlike animals, plants and fungi are largely sessile, and can not move to protect themselves from natural enemies. Instead, they produce a suite of secondary metabolites, ranging from caffeine in coffee, to psilocybin in magic mushrooms, to the spice in chili peppers. Humans have long exploited such compounds for medical use. However, little work has been devoted to understanding the role such secondary chemistry plays in the natural environment. Here, we explore the medical and ecological role of secondary metabolites of a notorious fungus, the death cap mushroom, *Amanita phalloides*. We first synthesize the medical research, digging deeper into the molecular mechanisms of treatments for amatoxin poisoning, and use these mechanisms to evaluate some of the conflicting medical advice surrounding amatoxin treatment. We then present an extraction protocol that saves time, reduces equipment contamination, and minimizes risk to the researcher. The impact of this faster, safer method may help produce these important toxins faster, for both research and medical use. We also examine toxin levels across several scales in California. Levels of alpha-amanitin are on the high end of published European values, indicating that either this invasive species underwent a genetic bottleneck from an area of Europe with high toxin levels, or *A. phalloides* may be under active selection to increase toxin levels in its introduced range.

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Acknowledgements

Scientific collaborations:

First and foremost, I wish to thank my Thesis Committee for shaping me as a scientist. My advisor Tom Bruns was always there for me when I had questions, and was especially supportive during the last few months of my dissertation, spent sheltering-in-place. John Taylor gives some of the best life advice. Anne Pringle has been my staunch supporter for 8 years, and I will always be her staunch supporter. Thanks to Britt Koskella for draft feedback as well as for her lively presence on Twitter. Among the Bruns-Taylor labs, in particular I want to thank Rachel Adams, Cheng Gao, Else Vellinga, Sydney Glassman, Christopher Hann-Soden, Iman Sylvaine, and Akiko Carver.

Among my peer scientific collaborators, I thank Scott Behie for being a supportive and fun science-friend. I look forward to more trips to Disneyland with you over our scientific careers!

Candace! I wish I'd met you my first year before starting all the toxin extractions. I'm so proud of the work I got to do with you, both in terms of research and outreach. You helped shape my papers to be so much better.

To my early LC-MS hookup and help collecting death caps, thank you to Nick Harris.

To my later HPLC hookup and friend on Animal Crossing, three cheers for Jackie Blake-Hedges.

To the wonderful undergrads who helped along the way, thank you: Jacob Baker, Sean Brown, Stephen Shen, Claudia MacFarlane, Gabriela Olea-Vargas, and Matthew Ricci.

Thank you to everyone that makes the annual Mendocino foray such a fun and informative experience, even if I did break my ankle that one time.

I also want to thank various Mycological Societies for talk invitations and awards that inspired me to continually evolve my story. Citizen scientists ask the best questions!

Friends and family:

I would never have been able to accomplish what I did without the support of a varied cast of friends and family. Foremost I thank my fiance, Mitchell Thompson. He keeps me fed, helps me with data analysis and Illustrator, and generally keeps me happy. Maxwell Crowsbane Fuzzyface also deserves a shoutout for keeping me happy. He was the best cat ever.

Thank you to my sister Shauna Adams for the endless online support, especially all the Tiger King memes during COVID-19 lockdown.

Simon I have known since middle school, and since we reconnected during undergrad, they have been a stellar best friend, supporting me from afar for years. I thank them for all the times they listened to me vent about critical feedback, heard me out when UBP talks were tough, and hosted me for trips back to Seattle to reconnect with friends and family.

I cherish memories with my friends and former housemates, Amy Spierling, Laura Kogler, and Anna Schneider. I will fondly remember dinners in the backyard,

cards, and watching Steven Universe on the projector. They also kept me fed, especially when I broke my ankle on that ill-fated mushroom collecting trip.

To my friend, my partner in crime, the co-parent of our immaculate love child, the Unconscious Bias Project: Linet, thank you for everything. Thank you to the entire host of characters who helped make UBP into a successful non-profit, most notably Mitch and Leigh during the early days, and also Simca, Theresa, Anna, Emily, Aaron, Tanuj, Lucrecia, Selena, Seth, Owen, Saffy and Destiny.

Thank you to Lanie for all the rides and general help, especially when Max got old and blind.

Shout-out to Maggie and Steven for all the fun doing mini golf or bowling, or Paint with Bob Ross and Pour Painting. Sometimes it's nice to use non-sciencey parts of my brain!

I want to acknowledge my parents, Ross and Linda, for always loving me for who I am, and special thanks to my dad for reminding me to slow the heck down.

Thank you to my sister Puck, my niece Gracie, my Aunt Jean, and Grandma for visiting me and giving me an excuse to do touristy things.

Thank you to my family-in-eventu-law: Mama T, Dad T, and sister Mary for giving me a second home and a second family. Props to Mary for also helping me out during my early days in AC:NH.

Grab-bag science thanks: Pablo Cruz-Morales, Chris Eiben, Bob and Maren. Among my cohort, thanks to Claire Willing, Norma Morella, and Carly Weiss.

Miscellaneous:

When I felt low, I found a lot of solace with video games, and especially with Twitch streamers like Billy1Kirby who offered virtual community. I also want to thank Twitch streamer MaxxxMurphy and his wholesome chat room for their evening company during the last 9 months of my dissertation. Thank you to games that allowed me to escape when I needed it, most notably Stardew Valley, World of Warcraft, and Animal Crossing: New Horizons. Animal Crossing was the virtual sociality I needed during the last few months of writing from lockdown.

I wish to thank many musicians for keeping me focused during my lab work and thesis writing. Notably, Mindless Self Indulgence, Rhapsody of Fire, Sonata Arctica, Nightwish, Children of Bodom, Rasputina, In Flames, Cradle of Filth, and the Crüxshadows.

I also want to thank a few medical professionals. Thank you to my therapist, Dr. Alicia del Prado for believing that I was physically sick even when all the early bloodwork came back "normal." My ever-lasting thanks to Dr Bonilla for always being willing to try new treatments, and for finding a cocktail that made my last year so, so, much easier than it would have been with my former energy levels. Thanks to Dr Mann for taking a scientific approach to putting my ankle bones back together, and for putting my ankle bones back together as well as could be expected.

Lastly, thank you to Tom Volk for helping me come to terms with being ill, and for some of the best encouragement I have ever received. I do hope mycology needs me!

Introduction

0.1. The natural role of secondary metabolites

Unlike animals, plants and fungi are largely sessile, and can not move to protect themselves from natural enemies. Instead, they produce a suite of secondary metabolites, ranging from caffeine in coffee, to psilocybin in magic mushrooms, to the spice in chili peppers. Humans have long exploited such compounds for medical use. However, little work has been devoted to understanding the role such secondary chemistry plays in the natural environment. Here, we explore the medical and ecological role of secondary metabolites of a notorious fungus, the death cap mushroom, *Amanita phalloides*.

This dissertation was compiled in the context of a global pandemic caused by the novel coronavirus COVID019, which has impacted all aspects of research, medicine, and everyday life.

0.2. Dissertation organization

Chapter 1: A review of *Amanita phalloides:* Species description, ecology, toxicology, and summary of amatoxin poisoning treatment

Focus:

Here we review the pertinent literature on *Amanita phalloides,* with a focus on its toxins and the role the toxins play in human health. The majority of this text will be included in a large collaborative review on all 29 known deadly poisonous mushroom species, to be published in *Fungal Diversity*.

Research Aims:

- Explore mycological taxonomic terminology
- Highlight the habitat and distribution of this species
- Describe amatoxins and phallotoxins
- Chronicle the typical symptoms of amatoxin poisoning
- Compare and contrast common treatments for amatoxin poisoning
- Outline potential research avenues of this fungus

Value:

While many reviews on amatoxin treatment exist, such reviews often cite studies without rigorously evaluating the merits of each study. Here, we synthesize the medical research, digging deeper into the molecular mechanisms of treatments for amatoxin poisoning, and use these mechanisms to evaluate some of the conflicting medical advice surrounding amatoxin treatment.

Chapter 2: A simple, fast, & efficient method to extract amatoxins from the death cap mushroom, *Amanita phalloides*

Focus:

This work was largely inspired by our collaboration with Candace Bever, a toxicologist at the USDA. In 2017 Candace began work to develop a lateral flow test to detect amatoxins. She sought collaboration in order to acquire mushrooms that contain amatoxins for her to test her antibodies. During the course of her work, she discovered she could remove enough amatoxin from a mushroom for her ELISA to read a positive result by merely hole-punching a piece of mushroom and hand-shaking it in water or PBS buffer, for a minute. This finding caused us to realize that the toxin extraction protocol we'd been using for years was likely overly complicated. Armed with that knowledge, we sought to show that recent protocols for extracting amatoxins from mushrooms like *Amanita phalloides* could be simplified.

Research Aims:

- Determine how much incubation time matters for extraction
- Determine whether we can minimize risk to researcher by simply hand shaking mushroom pieces
- Compare evaporating solvent with either a rotovap (one sample at a time) vs speedvac (several samples simultaneously)

Value:

We present an extraction protocol that saves time, reduces equipment contamination, and minimizes risk to the researcher. We also discuss what may be the first comparison of rotovap and speedvac methods, finding that speedvac may save time, but, depending on how the sample is treated, can lose toxin relative to rotovap. The impact of this faster, safer method may help produce these important toxins faster, for both research and medical use.

Chapter 3: Amatoxin and phallotoxin levels of *Amanita phalloides* in California across multiple scales: within a mushroom, between sites, and over time

Focus:

My previous work with wild chili peppers attempted to answer the question "Why are chilies hot?" In a similar vein, I set out to answer data that would help answer the question, "Why is the death cap mushroom poisonous?" This fungus, as an introduced species in California, is an ideal system for understanding the role toxins could potentially play in a range expansion. However, most of the previous work done to measure toxins in *A. phalloides* has been performed on European samples. In order to answer the role the toxins play in its invasion success, we first must understand toxin levels in California.

Research aims:

- Determine whether toxin concentration varies within a single pileus
- Determine whether toxin concentrations vary across the different organs of an individual mushroom (pileus, stipe and volva)
- Quantify how much toxin concentrations differ between established sites in a native forest
- Determine whether toxin concentration differs between years at the same site

Value:

Here we use the exact same extraction protocol on many dozens of mushrooms collected from native California forest. Very little work has quantified toxin levels of mushrooms collected in the introduced range of *Amanita phalloides*, and rarely has anyone attempted to frame questions in a way that tests ecological theory. Furthermore, because the native status of *Amanita phalloides* was uncertain for many years, this is perhaps the first work to test toxin levels in a framework that reveals something about how the toxins may be changing relative to its natural range in Europe. We anticipate the story will be further enriched when quarantine is lifted and the samples extracted over the last year (California samples from 2017, Portuguese samples from 2018) can be included in the analysis.

Chapter 1. A review of *Amanita phalloides:* Species description, ecology, toxicology, and summary of amatoxin poisoning treatment

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1.1. Introduction

To understand the ecological role of deadly mushroom toxins, one must understand the role these toxins play in poisoning humans. This chapter will begin with a taxonomic description of the death cap mushroom, *Amanita phalloides*. Following the taxonomic review, we will explore its ecology and distribution. Next, we will introduce the toxins found in each species, and provide information on how they function at the molecular level. Molecular information will be followed by a treatise on how amatoxins cause poisoning in humans. Several case studies will be followed by an overview of the current status of medical treatment of amatoxin poisoning. Lastly, several potential directions for future research will be discussed.

1.2. *Amanita phalloides* (Fr.) Link HandbuchzurErkennung der nutzbarsten und am häufigstenvorkommendenGewächse: 272 (1833)

1.3. Taxonomic description

Robust basidioma with pileus, stipe, lamellate hymenium, and both universal and partial veils. The following description is based on those by Ammirati et al and Siegel (Ammirati et al. 1977) (Siegel 2016). *Pileus* reaching 30–150 mm in diam. at maturity, first egg shaped and tightly convex, expanding to broadly convex to subumbonate, margin often paler than center, straight to incurved, rarely striate, color variable, often greenish yellow to gray green, sometimes with an olive, metallic tint, sometimes darker streaks, occasionally fading to pale yellow or becoming brownish yellow to medium brown; occurs rarely as an all-white variation, named *Amanita phalloides* var. *alba*; surface smooth, slightly viscid when young, becoming silky, usually glabrous with occasional white patches of volva; context 2–6 mm thick, usually white except when yellow immediately below the peelable cuticle, color unchanging on bruising. *Lamellae* free or finely attached, white to creamy white, sometimes becoming very pale yellow with age, unchanged when bruised, close to crowded, ventricose, with several layers of unevenly distributed, truncate lamellulae. *Stipe* 50–150 × 10–30 mm, usually tapered towards the apex from a bulbous base, upper portion white to creamy white or pale yellow, lower portion usually darker, sometimes yellowish green, unchanging or becoming more yellow when bruised, surface usually dry, often glabrous and with darker zig-zag markings, context soft and cottony, pulling apart in longitudinal strips (personal observation), stuffed at apex end but often hollow toward base, base solid, variably white, unchanging when exposed. *Annulus* apically attached, thin, membranous, a white or creamy white hanging skirt, sometimes collapsing against stipe. Usually persistent, but occasionally disappearing, becoming powder-like or remaining attached to cap margin. *Volva* thin, white, well developed and

spacious). *Taste* and *odor* of younger specimens pleasant, older basidiocarp odor like rotting potatoes. *Taste* mild, indistinctive. *Spore print* white.

Basidiospores (7–)8–10(–12) × (5.5–)6–8(–10.5) µm, subglobose to ellipsoid, smooth, hyaline in KOH and amyloid in Melzer's; apiculus broad and eccentric. *Basidia* 50–60 × 8–12 µm, 2- to 4-spored, clavate, with long sterigmata, thin-walled, hyaline in KOH. *Pleurocystidia* absent. *Cheilocystidia* apparently absent, but sterile marginal cells along lamellar edges often abundant, saccate, broadly clavate, clavate or subcylindrical, 27–63 × 7.2–36 µm, thin-walled, smooth to slightly roughened, hyaline in KOH, some containing colorless to yellowish droplets and granules, nonamyloid.

1.4. Distribution, habitat, and ecology

Amanita phalloides is native to central Europe (Watling 1984), extending as far southwest as Portugal (Garcia et al. 2015; Kaya et al. 2013) and reaching South East as far as Macedonia and Bulgaria (Marinov et al. 2018). It is also known in North Africa (Malençon & Bertault 1970). The death cap is abundant as far East as Turkey (GÜRBÜZ et al. 2015; Durukan et al. 2007), but the extent of its Eastern range remains poorly known to westerners. The northern extent is determined by the occurrence of *Quercus* in Scandinavia (Lange 1974).

Though the native presence of *A. phalloides* in Europe has been well established for hundreds of years, until the beginning of this century, the scientific literature was inconsistent regarding its native status outside Europe. Three primary reasons contributed to this confusion. One complication was the lack of dried European specimens outside Europe to compare specimens against, as well as a dearth of individuals who had seen *A. phalloides* in its native range and also traveled to positively identify it elsewhere (Tanghe and Simons 1973). The second, more problematic issue, was the use of a very general species concept, which allowed for multiple species to be included within the supposed description for *A. phalloides* (Tanghe and Simons 1973; Pringle and Vellinga 2006). Lastly, a pure white variety, *A. phalloides var. alba* (Kaya et al. 2013), allowed for other pure white *Amanita* species such as *A. bisporigera* and *A. ocreata* to be misidentified as *A. phalloides*.

In North America, by the 1930's, some mycologists noted that *A. phalloides* grew commonly throughout the US (Lange 1934). But other authors doubted or flat-out denied the true European, olive-green *A. phalloides* was really present in North America (Tanghe and Simons 1973). An in-depth literature search was designed to determine whether *A. phalloides* was in fact a true native to North America, or rather an interloper accidentally re-located with European seedlings. The literature was consistent with respect to the death cap being introduced on the East coast of the USA, but could not determine the native status of death caps on the west coast (Pringle and Vellinga 2006).

To verify if *A. phalloides* was introduced to California from Europe, Pringle et al annotated 11 California herbarium specimens collected between 1911 and 1962 (Pringle et al. 2009). By sequencing the ITS region, which was found to be invariable in *A. phalloides*, it became clear that all herbarium specimens labeled as *A. phalloides* prior to 1938 were actually other *Amanita* species. The first true recorded *A. phalloides* was collected in 1938 on the Del Monte Hotel grounds (now Naval Postgraduate School), near Monterey, California. Records show *A. phalloides* was common in Delaware, New Jersey

and New York by 1970 (Tanghe and Simons 1973). Today, the death cap continues to expand its range into the Simon Fraser River Valley in Vancouver Island, British Columbia, Canada (Berch et al. 2017; Moor-Smith et al. 2019). There, this promiscuous invader is associating with another native tree, *Quercus garryana*, British Columbia's only native oak species (Berch et al. 2017).

The death cap was frequently found outside North America as well, likely dispersed with *Eucalyptus* and *Pinus* species. In South Africa, Dutch settlers planted European oaks around their farms, and soon-after noticed *A. phalloides* fruiting (Mikola 1969). *Amanita phalloides* has been found in Eucalyptus tree plantations in Africa and Madagascar (Ducousso et al. 2012), and it thrives in pine plantations (van der Westhuizen and Eicker 1987), or with introduced trees such as *Quercus* and *Populus* (Mikola 1969). By the 1950s, the death cap was common in pine plantations in South America (Dunstan et al. 1998), such as in Argentina, associating with *Pinus radiata* (Mikola 1969), and in Uruguay growing with introduced pine (Malajczuk et al. 1982). Death caps are also common in *Pinus* plantations in New Zealand (Ridley 1991; Dunstan et al. 1998) and Eastern Australia (Shepherd and Totterdell 1988; Dunstan et al. 1998).

Amanita phalloides is a generalist ectomycorrhizal species. It associates with both evergreen species in the *Pinaceae* as well as deciduous and evergreen hardwood trees such as those in the genus *Quercus* (Wolfe and Pringle 2012), and can be found in mixed conifer and deciduous forests. In Europe, the death cap usually begins fruiting after the first few autumn rains and continues fruiting through the early winter. In California, USA, it can be found throughout the year (Wolfe and Pringle 2012), especially in watered areas such as botanical gardens and university campuses. The death cap can fruit singly, in small groups, or in luxuriant patches of several dozen, the latter being especially common along roadsides and other disturbed areas, particularly in its introduced range (Tanghe and Simmons 1973).

Thus far, little work has examined how *A. phalloides*' growth habits and ecology may differ in both native and introduced ranges. Wolfe et al. found that California basidiocarps are often more than twice as large as European mushrooms (Wolfe and Pringle 2012). The authors also showed that in California the death cap selectively associates with the widely occurring California live oak, *Quercus agrifolia*, despite an array of other supposedly suitable tree hosts. In contrast, on the North American East Coast, its host range is more restricted, and it more commonly associates with pines (Wolfe and Pringle 2012). The precise reason for this host specificity in its introduced range remains unknown.

Figure 1-1 Young *Amanita phalloides* specimens from Santa Cruz County, California. Photo Credit: Christian Schwarz.

Figure 1-2 Young and mature *Amanita phalloides* specimens from Santa Cruz County, California. Photo Credit: Christian Schwarz.

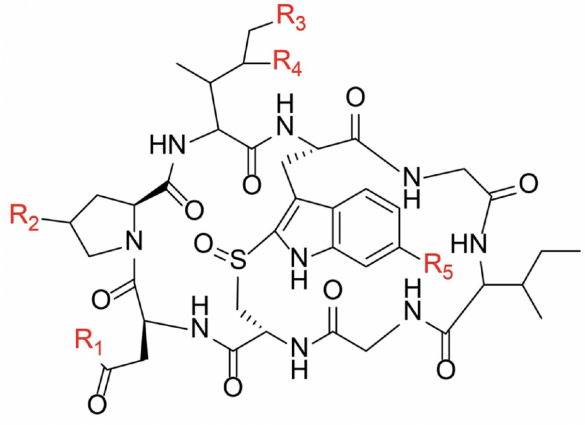
1.5. Amatoxins & phallotoxins

1.5.1. Amatoxins

Nine amatoxins are known to science, three of which are well studied: α-amanitin, β-amanitin and **γ**-amanitin; the remaining six are amaninamide, amanulin, proamanulin, amanin, ε-amanitin and amanullinic acid. Unlike many fungal secondary metabolites, the amatoxins are produced on ribosomes, and are not synthesized by a non-ribosomal peptide synthetase (NRPS). The amatoxins are cyclic octapeptides composed solely of L-amino acids (Wong and Ng 2006), and the various toxins differ with respect to hydroxylations of the side chains (**Figure 3**). Only amanullin and proamanullin are nontoxic to humans, while the remaining seven are known to be deadly to humans (Wieland and Faulstich 1978).

Amatoxins allosterically inhibit RNA polymerase II (pol II), which transcribes mRNA as well as miRNA, snRNA and snoRNA (Carter and Drouin 2009). Amatoxins specifically inhibit the translocation step (Gong et al. 2004); the alpha-amanitin binding site is located in a funnel-shaped cavity, beneath a bridge helix that spans the gap between the two largest pol II subunits, Rpb1 and Rpb2 (Bushnell et al. 2002). Once bound to RNA pol II, alpha-amanitin quickly and efficiently shuts down protein production. Early reports found

amanitin also fragments the nucleolus, the site of ribosome biogenesis, and causes early condensation of chromatin (Fiume et al. 1969), which may contribute to its toxicity.



Toxin	R_1	R_2	R_3	R_4	R_5
α-amanitin	NH_2	OH	OH	OH	OH
amaninamide	NH_2	OH	OH	OH	H
γ-amanitin	NH_2	OH	OH	H	OH
amanullin	NH_2	OH	H	H	OH
proamanullin	NH_2	H	H	H	OH
β-amanitin	OH	OH	OH	OH	OH
amanin	OH	OH	OH	OH	H
ε-amanitin	OH	OH	OH	H	OH
amanullinic acid	OH	OH	H	H	OH

Figure 1-3 Structure of amatoxins.

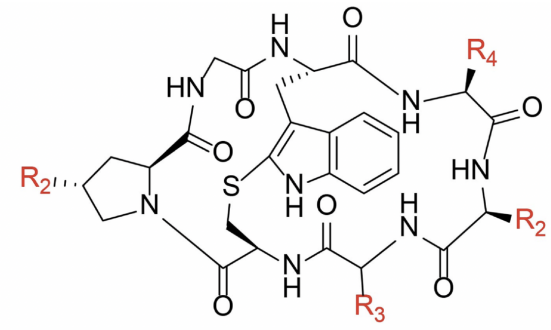
Once inside the human liver, all nine of the amatoxins bind to the RNA pol II of hepatocytes, inhibiting transcription and ultimately resulting in cell death. It is estimated that alpha-amanitin concentrations as low as 3×10^{7} M can block 90% of transcription activity (Mengs et al. 2012). The toxic effects are enhanced by enterohepatic circulation (Wieland and Faulstich 1991), i.e., in which toxin that is absorbed by the small intestine is removed from the intestine by the liver and sent to the gall bladder where it, along with bile salts, is returned to the small intestine to again cycle through the liver, inflicting more and more damage with each passage. If the patient is insufficiently hydrated, amatoxins can also accumulate in the kidneys, causing lesions and often resulting in death due to hepatic and renal failure (Mengs et al. 2012).

Amatoxin poisoning is characterized by four main phases (**Table 1**): 1): an asymptomatic lag phase; 2) a gastrointestinal phase featuring symptomatic presentation; 3) a honeymoon phase featuring apparent recovery despite ongoing liver damage; and 4) acute liver failure in which a build-up of toxins begins to impact brain and kidney function, potentially leading to fulminant organ failure and death.

Table 1-1 Main phases of amatoxin poisoning.

Phase	Information	Reference
Lag phase	Incubation period lasts 6–40 hours, with an average of 10 hours.	(Santi et al. 2012)

Gastrointestinal phase	When a patient finally seeks medical attention, the amanitins have been crippling protein synthesis in the liver for hours. Eventually, the patient begins to suffer vomiting, abdominal pain, and diarrhea.	(Ward et al. 2013)
Honeymoon phase	24–36 hours after ingestion, some patients experience a phase of apparent convalescence. Outward symptoms such as diarrhea abate for several hours while the toxins continue to damage the internal organs. Levels of serum transaminases and lactic dehydrogenase are still high, but until the patient shows signs of jaundice, the continual liver damage might go unnoticed.	(Santi et al. 2012);(Vo et al. 2017)
Acute liver failure	4-9 days after ingestion: If left untreated, the transaminase levels continue to rise while liver and renal functions deteriorate.	(Mengs et al. 2012; Santi et al 2012)



Toxin	R_1	R_2	R_3	R_4	R_5	R_6
phallisin	CH_3	CH_3	OH	OH	OH	OH
phalloidin	CH_3	CH_3	OH	H	OH	OH
phalloin	CH_3	CH_3	OH	H	OH	H
prophalloin	CH_3	CH_3	H	H	OH	H
phallisacin	$CH(CH_3)_2$	COOH	OH	OH	OH	OH
phallacidin	$CH(CH_3)_2$	COOH	OH	H	OH	OH
phallacin	$CH(CH_3)_2$	COOH	H	OH	H	OH

Figure 1-4 Structure of phallotoxins.

1.5.2. Phallotoxins

Phallotoxins are related to amatoxins, similarly produced on the ribosome, and are bicyclic heptapeptides (**Figure 4**). Seven phallotoxins have been described, but like the amatoxins, more are likely awaiting discovery and description (Sgambelluri et al. 2014). Phalloidin (Ala-Trp-Leu-Ala-D-Thr-Cys-Pro) and phallacidin (Ala-Trp-Leu-Val-D-Asp-Cys-Pro) are usually considered the main amatoxins and are the only ones for which commercial standards are available. The others, including phallisin, phalloin, prophalloin, phallisacin and phallacin, occur in smaller concentrations and are thought to be less bioactive.

Phallotoxins are less toxic than amatoxins, and in eukaryotes bind actin (Wieland and Govindan 1974), the major microfilament cytoskeleton protein. Phallotoxins also bind actin-related proteins in prokaryotes (Guerrero-Barrera et al. 1996). Once bound,

phallotoxins prevent the polymerization of F-actin, thus inhibiting cell motility and muscular contraction, and altering cell shape (Guerrero-Barrera et al. 1996). Unlike the amatoxins, which are quickly absorbed in the intestines of most mammals, animal studies reveal phallotoxins are never absorbed (Wieland and Faulstich 1978).

1.6. Amatoxin poisoning

Amanita phalloides was recently categorized as the 7th most feared fungus on Earth, the only mushroom among fungal threats such as human pathogens and *Aspergillus flavus*, the primary producer of aflatoxins (Hyde et al. 2018). The death cap's victims reach back hundreds, and likely thousands, of years. The list of victims potentially includes people of historical significance such as Roman Emperor Claudius (Marmion and Wiedemann 2002). Cooking does not render the mushrooms safe, making them dangerous for both accidental and intentional food poisoning (Jansson et al. 2012). No known enzyme can degrade either amatoxins or phallotoxins, and both toxin families can be boiled for several minutes without being denatured (Wieland and Faulstich 1978). For example, in one instance, mushrooms that were dried, frozen for 7 months, and finally cooked before consumption remained potent, killing the patient (Himmelmann et al. 2001).

A few papers have examined the varying toxin levels in its native and introduced ranges. In the United States, death caps collected in Oregon and Washington were measured as having lower toxin levels than mushrooms from Europe (Tyler et al. 1966), though these values were calculated from Thin Layer Chromatography and quantified based on a single standard. The amount of amatoxin in a mature fruiting body can range from 0.88 to 2.95 mg/g dry weight (Sgambelluri et al. 2014; Garcia et al. 2015).

Animal studies informed much of our knowledge on the mechanism of action of amatoxins. In animals such as dogs, cats, and guinea pigs, the toxin is absorbed in the gastrointestinal (GI) tract, meaning an animal can die of doses of only a few mg, when administered per-orally. Rabbits are immune when the toxin is administered orally, presumably because in rabbits the toxins are not absorbed by the GI tract (Wieland et al. 1954). In mice, the LD_{50} is ~0.3 mg/kg body weight, while rats have an LD_{50} of 4 mg/kg, and dogs, like humans, are more sensitive with an injected **LD_{50}** of only 0.1 mg/kg (Wieland and Faulstich 1978).

Much of the confusion over the effectiveness of purported treatments for *A. phalloides* poisoning stems from the model systems used, and how the data from such studies were interpreted. Unlike mice and rats, dogs experience amatoxin poisoning symptoms that more closely mirror those of humans. Dogs experience early dehydration, vomiting, and hypoglycemia, and later hepatic lesions develop, as evidenced by elevated liver enzymes (Vogel et al. 1984). Dogs also succumb to amatoxin much later than mice, after about 30-40 hours (Floersheim et al. 1978). Therefore, compared to mice, dogs are much better model systems for understanding how a treatment may work in humans.

However, while dogs appear to be the closest animal model to humans for testing amatoxin treatments, they have limitations. In a study using cultured canine hepatocytes, Magdalan et al found that neither penicillin, silibinin, nor acetylcysteine had any measurable effect on reducing cytotoxicity from amanitin (Magdalan et al.

2009). However, in a study with human hepatocytes and a lower dose of amatoxin, all three treatments conferred protective effects (Magdalan et al. 2010). It should be noted that hepatocytes can not undergo enterohepatic recirculation. These contrasting results may be due to the limits of a cellular model, interspecies differences, or the higher amatoxin dose used in the canine study (Magdalan et al. 2010). Despite not allowing for the testing of treatments on enterohepatic recirculation, human hepatocytes may be a useful, animal-free way of testing the efficacy of various treatments.

In humans, the main mechanism of toxicity occurs from hepatocellular uptake, as the toxins enter the liver via the organic anion–transporting polypeptide (OATP) receptor family (Letschert et al. 2006). The sodium-dependent bile acid transporter Na+-taurocholate cotransporter polypeptide (Ntcp) also seems to play a role in amanitin uptake by the liver (Gundala et al. 2004).

1.7. A sampling of medical case studies from around the world

1.7.1. Case study 1

In Switzerland, a 61-year-old female patient was hospitalized with severe dehydration due to vomiting and diarrhea and she reported ingesting several mushrooms about 36 hours earlier. She had picked the mushrooms the previous autumn (about 7–8 months earlier), dried them, and kept them in the freezer. She had cooked the mushrooms before eating the entire batch. The diagnosis of *A. phalloides* poisoning was confirmed by detection of amatoxin in the urine at a level of 37.3 µg/l (measured approximately 4 days after ingestion). The patient declined evaluation for a liver transplant and died on day 4 from progressive liver and renal failure (Himmelmann et al. 2001).

1.7.2. Case study 2

Four cases of *Amanita phalloides* poisoning were reported from Portugal, two of which resulted in fulminant hepatic failure and required orthotopic liver transplantation (Alves et al. 2001).

1.7.3. Case study 3

In the fall of 1981 in San Francisco, a pronounced increase in the number of mushroom-related calls was noted. From October 1 through mid-December, 96 calls were received, a fivefold increase over the same period in 1980. Among these were ten cases of presumed *Amanita phalloides*-type poisoning, of which three were fatal (Olson et al. 1982).

1.8. Types of Amatoxin Treatment

The approaches to treating amatoxin poisoning are many, controversial, and still evolving to this day. The relative rarity and immediate danger of the toxins necessitates multiple, concurrent treatments. While well-meaning, such approaches have confounded our understanding of the precise contribution of each individual antidote.

We will begin with the most promising, effective treatments, and then discuss several commonly used ineffective treatments.

1.8.1. Silibinin and penicillin

An obvious treatment would be to interrupt enterohepatic recirculation, and end the uptake of amatoxin into the liver. Extracted from seeds of the milk thistle *Silybum marianum*, silibinin (Vogel et al. 1984) (**Figure 5**), administered intravenously (IV), strongly inhibits the hepatocyte uptake of amatoxin by competitive inhibition of the Organic Anion Transporting Polypeptides (OATP) transporters (Faulstich et al. 1980; Letschert et al. 2006). This inhibition occurs during both the primary and the enterohepatic circulation of the toxin (Mengs et al. 2012). The administration of silibinin is the standard of care for amatoxin treatment in Europe (Roberts et al. 2013) and the only amatoxin-treatment that has been the focus of a clinical trial in the United States (Mengs et al. 2012).

The amatoxin-protective properties of IV silibinin have been well demonstrated in both animal and human studies. A study with beagles found that none of the 13 dogs treated with IV silibinin died, while 4 of the 12 untreated dogs died (Vogel et al. 1984). The silibinin-treated dogs had lower gastrointestinal distress and much less pronounced liver damage, as measured by levels of serum transaminases (AST, ALT), bilirubin and prothrombin time. A retrospective study of 18 human cases found a close relationship between the severity of liver damage and the delay between mushroom ingestion and onset of silibinin treatment (Hruby et al. 1983). Except for one suicidal individual who consumed an extremely high dose of mushrooms, all patients treated with IV silibinin survived.

The inherent danger of amatoxins prohibits any sort of voluntary study with humans, but one can gather data on patients as they present to a medical facility for treatment. Mengs et al found that, with an *n* of nearly 1,500, the mortality in patients treated with aggressive hydration coupled with IV silibinin (Legalon® SIL) was less than 10%, compared to over 20% when using penicillin alone or a combination of silibinin and penicillin (Mengs et al. 2012). These findings mirror those of a retrospective analysis in Germany, which found that only 5.1% of patients who received silibinin alone died or underwent liver transplantation, compared to 8.8% of patients who received a combination of silibinin and penicillin (Ganzert et al. 2008).

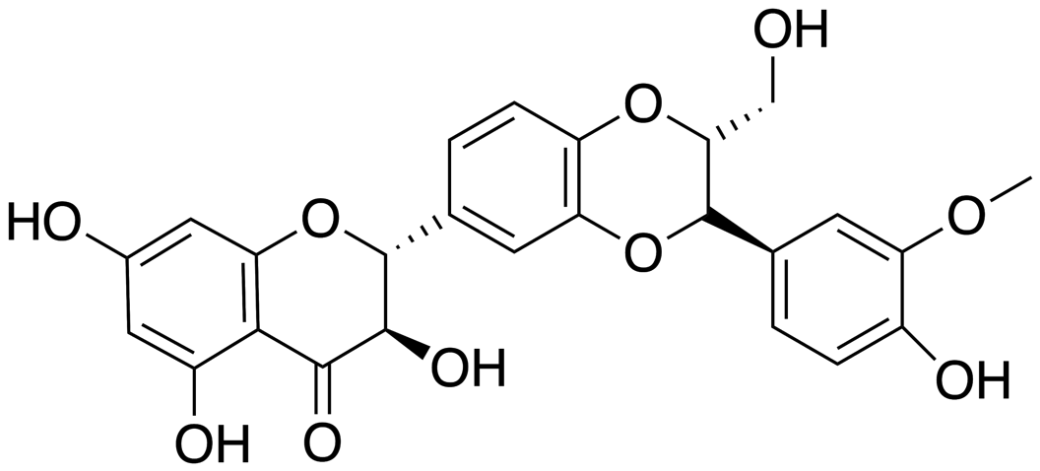


Figure 1-5 The chemical structure of silibinin, the primary isomer of silymarin.

Penicillin (also called benzylpenicillin and Penicillin G) seems to have a similar mode of action as silibinin, likely binding amatoxins or preventing amatoxin uptake by hepatocytes (Faulstich et al. 1980). However, the ability of penicillin to block uptake is thought to be less than that of silibinin (Wieland 1986). In dogs, a high dose of penicillin was effective in suppressing liver enzyme levels (Floersheim et al. 1978), indicating decreased liver damage. Future work could explore the pharmacokinetics/pharmacodynamics (PK/PD) of penicillin when combined with silibinin: perhaps the presence of penicillin causes upregulation of processes that degrade or otherwise inhibit silibinin. The clinical data indicate penicillin can be a second-tier treatment when silibinin is unavailable.

In treating any poisoning, silibinin is far more effective when administered intravenously compared to orally. Orally, it has low bioavailability due to poor water solubility, and can only reach a fraction (1.13–1.33 ug/ml) of the maximum plasma concentration of IV silibinin (6.04 ug/ml) (Kim et al. 2003).

1.8.2. Aggressive hydration and biliary drainage

Preliminary data from a silibinin clinical trial conducted in the US (Mengs et al. 2012) found that silibinin does not protect the kidneys. Historically, more attention has been given to liver failure than kidney failure, but the trial emphasized that as long as the patient is sufficiently hydrated, functioning kidneys can effectively clear circulating amatoxins, and this finding was confirmed by trials conducted in India (Mengs et al. 2012). Many of the benefits of purported antidotes may simply stem from coupling antidotes with a simple, more effective treatment: aggressive hydration.

The clinical trial conducted in the US also showed that, when silibinin is unavailable, draining the biliary tract to externally remove amatoxins may be an effective alternative treatment (Mengs et al. 2012). Biliary drainage efficiently removed amatoxins from beagles fed the Chinese amatoxin-containing mushroom *Amanita exitialis* (Sun et al. 2018). Ultrasound-guided gallbladder aspiration is similarly helpful in humans and is

minimally invasive, technically straightforward and quickly accomplished (Anupam and Kumar 2017).

1.8.3. Liver transplant

Liver transplant may be necessary when patients consume a large number of mushrooms, but transplant does not always save the patient (Bernuau et al. 2005; Ganzert et al. 2008; Mas 2005). Furthermore, liver transplant is not always an option on short notice. Recall the amatoxins recycle with bile salts, and the human body has no way of breaking down or otherwise reducing the effect of amatoxins. If the patient is aggressively hydrated to the point of diuresis, large amounts of amatoxins can pass through the kidneys and be dispelled in urine (Pinson et al. 1990; Mengs et al. 2012). However, preparation for liver transplant usually involves limiting fluid intake to decrease intracranial pressure to prevent encephalopathy (Pinson et al. 1990; Sun et al. 2014). Such measures decrease the patient's urine output and limit toxin removal through the kidneys. In these cases, the new liver can be re-poisoned by recirculating toxins.

1.8.4. Lesser treatments

Some drugs continue to be used despite their dubious contribution to alleviating amatoxin poisoning. The delayed symptoms of amatoxin poisoning render treatments like activated charcoal (AC) largely ineffective, as AC absorbs toxins and is best administered within 30-60 minutes of ingestion (Bond 2002). After 180 minutes, absorption drops to 21.13%, and ceases to be clinically relevant (Chyka et al. 2005). The mushroom has usually vacated the stomach many hours prior to a patient arriving at a medical facility. Even if AC were administered immediately, volunteer studies show AC does not improve patient outcomes for any toxin (Chyka et al. 2005), yet it is still ubiquitously administered to treat poisonings of many kinds.

N-Acetylcysteine is an extremely commonly used antidote, being the primary method to reverse poisoning from acetaminophen (brand name Tylenol), the worldwide leading cause of drug overdose and acute liver failure (Bunchorntavakul and Reddy 2013). Acetaminophen is metabolized to a reactive metabolite which depletes glutathione and covalently binds proteins (Hinson et al. 2010). In the case of acetaminophen poisoning, N-Acetylcysteine enhances nontoxic routes of acetaminophen metabolism, while in amanitin poisonings, N-Acetylcysteine is thought to reduce radical-induced injury and restore rodox capacity (Ward et al. 2013). In mice, it was not found to reduce hepatic enzyme elevation or improve survival (Schneider et al. 1992). Its precise mode of action in alleviating amatoxin poisoning in humans remains elusive, but the benign side effects and possible benefits usually justify the use of N-acetylcysteine despite its uncertain contribution (Ward et al. 2013). However, caution should be taken in administering this drug, as it can cause anaphylactic shock (Bailey and McGuigan 1998).

Cimetidine is a histamine H2-receptor antagonist (Macdougall et al. 1977) that inhibits stomach acid reduction by inhibiting cytochrome P450. Because of its non-selective inhibition, it has numerous drug interactions. Cimetidine was administered in a mice study because of the (erroneously) believed similarity of amatoxin poisoning to acetaminophen poisoning (Schneider et al. 1987). Amatoxins are not metabolized in the liver into toxic byproducts, unlike acetaminophen. This problematic study began with

two mice immediately dying from injection with cimetidine prior to any injection of alpha-amanitin (Schneider et al. 1987). After adjusting the dose, liver dissections purportedly showed cimetidine reduced hepatic injury from amanitin as measured by steatosis, the abnormal retention of lipids. As all mice were sacrificed after 48 hours, the contribution of this treatment to mouse survival remains unknown. Another animal in the control group died from unknown causes, calling further doubt on the dubious nature of this study's conclusions.

The choice of animal model greatly influences how the results of poisoning studies should be interpreted. Like humans, dogs experience early dehydration, vomiting, and hypoglycemia, and later hepatic lesions develop, as evidenced by elevated liver enzymes (Vogel et al. 1984). Dogs also succumb to amatoxin much later than mice, after about 30-40 hours (Floersheim et al. 1978). Using beagles as a model system, Floersheim et al 1978 found no favorable effects of cimetidine on gastrointestinal bleeding, biochemical regulation, or coagulation factors. Furthermore, in cimetidine-treated dogs, aminases and alkaline phosphatase tended to run higher compared to the control dogs (Floersheim et al. 1978). Cimetidine was used in a later murine model study (Tong et al. 2007), with similarly lackluster results.

Future clinical trials will be instrumental to untangle the conflicting medical advice surrounding the treatment of amatoxin poisoning.

1.9. Future directions

The death cap mushroom is both an invasive species and a potent source of secondary metabolites, rendering it a rich model organism to address ecological, genetic and metabolic research questions. For example, an ongoing Citizen Scientist project aims to collect data on whether *Amanita phalloides* exhibits less insect damage in its introduced range compared to its native range (iNaturalist 2009). Such a difference would indicate release from its co-evolved enemies has occurred (Colautti et al. 2004). The project also aims to answer if amatoxin-wielding *Amanita* species experience less insect damage than their non-toxic sister taxa. Such data will help answer what protective role, if any, amatoxins play in their natural environment.

Similarly, the natural intended target of both the amatoxins and phallotoxins remain unclear. However, resistance to amatoxins has been demonstrated in mycophagous *Drosophila* species (Greenleaf et al. 1979) and the nematode *Caenorhabditis elegans* which parasitizes *Drosophila* (Sanford et al. 1983). Future work could examine whether insects in its introduced ranges are more readily poisoned by amatoxins and phallotoxins than insects in its native range.

Amanita phalloides is also a useful system for studying fungal genetics, as few papers have investigated the genetics of introduced ectomycorrhizal fungi. A recent preprint analyzed AFLP and whole-genome sequencing to show the genet size of *A. phalloides* is quite small, and rarely consists of more than several mushrooms (Golan et al. 2019). This trend is true both in California, where *A. phalloides* is invasive, and in its native range of Europe. Furthermore, the genets appear to be short-lived, as the same genet was never found from one year to the next. Much work remains to explore which genes are under selection during its host shifts and range expansion.

The same amatoxins responsible for taking lives also have the potential to extend human life. Compared to healthy cells, cancer cells exhibit a more active RNA polymerase (Riede 2010). Due to the specific targeting of amatoxins against pol II, extracts of *A. phalloides* were used to suppress the MCF-7 breast cancer cell line (Kaya et al. 2014). When used in an antibody-drug conjugate, α-amanitin was successful in treating both drug-tolerant cancer cells as well as mice suffering cancer relapse (Kume et al. 2016). Of note is that the drugs used in studies like these are still extracted from *A. phalloides* mushrooms. A breakthrough occurred in 2018, as researchers finally synthesized alpha-amanitin chemically (Matinkhoo et al. 2018), which will open up doors for the future use of amatoxins in medicine and research.

Linking lab toxin research to their applications in medicine is not straight forward. One of the foremost difficulties lies in our present inability to rapidly and easily detect amatoxins. A collaboration with the USDA recently developed a lateral flow immunoassay (LFIA) to accurately and rapidly detect amatoxins from mushrooms (Bever et al. 2020), which will be a valuable tool for aiding medical professionals to select an appropriate treatment regime.

1.10. References

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Chapter 2. A simple, fast, & efficient method to extract amatoxins from the death cap mushroom, *Amanita phalloides*

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2.1. Abstract

The death cap mushroom, *Amanita phalloides*, is well known for containing amatoxins such as alpha- and beta-amanitin, which inhibit mammalian RNA polymerase II. While these toxins have been used in research for almost a century, they have recently garnered attention for their role in drug-antibody conjugates. The amatoxins are still largely extracted from wild mushrooms, which can not be grown in the lab.

We propose simplified extraction methods that could reduce hazardous exposures to dust, and expedite sample analysis without sacrificing accuracy. We recently developed a Lateral Flow Immunoassay (LFIA), for which we identified that sample maceration was not needed to extract the amatoxins and that the incubation time for extraction could be accomplished in 1 minute. In this current work, we hypothesized that these same extraction adjustments–minimal tissue maceration and reduced incubation time–could be transferable to instrumental detection methods.

To test the need for sample maceration, we utilized three different techniques: 1) traditional mortar and pestle, 2) a similarly disruptive method of bead beating, and 3) no grinding, but rather hand shaking dried mushroom tissue in the extraction buffer. In addition, we performed the solvent extraction step at varying times to observe if more time allows for more toxin to be removed from the tissue. Lastly, we utilized two comparable solvent evaporation methods (rotovap or speedvac) to establish if multiple samples could be processed simultaneously, thus improving sample throughput.

We adjusted aspects of the typical extraction protocol, which resulted in a rapid (1 min) incubation step, along with minimal sample handling (no grinding) of the dried mushroom tissue. We present an extraction protocol that saves time, reduces equipment contamination, and minimizes risk to the researcher. The impact of this

faster, safer method may help produce these important toxins faster, for both research and medical use.

2.2. Introduction

Wild mushrooms produce many compounds of biological significance (Lull et al. 2005; Holliday and Cleaver 2008; Jayakumar et al. 2008; Heleno et al. 2011; Alves et al. 2012; Heleno et al. 2013; Soares et al. 2013; Chang et al. 2015; Kozarski et al. 2015; Phan et al. 2015; Taofiq et al. 2016; Daley et al. 2017; Wasser 2017). One mushroom species of intense pharmacological interest is *Amanita phalloides*, also known as the death cap mushroom. The death cap is well known for producing the bicyclic octapeptides called amatoxins as well as the related heptapeptides the phallotoxins, which inhibit RNA polymerase (Wieland 1983; Carter and Drouin 2009) II and actin polymerization (Wieland and Govindan 1974; Vandekerckhove et al. 1985), respectively. Both toxins have been used in cell research for decades (e.g. (Schultz and Hall 1976; Wieland and Faulstich 1978; Warn and Magrath 1983; Jendrisak 1980; Anderl et al. 2012). Furthermore, the primary amatoxin, alpha-amanitin, has been found to have strong anti-tumor effects. It was effective against the MCF-7 breast cancer cell line (Kaya et al. 2014), and when used in an antibody-drug conjugate (Pahl et al. 2018), alpha-amanitin successfully treated both drug-tolerant cancer cells and mice suffering cancer relapse (Kume et al. 2016).

For the last sixty years, commercial standards of the primary amatoxins (alpha, beta and gamma-amanitin) and primary phallotoxins (phalloidin, phallacidin) have been largely extracted from wild-foraged mushrooms, primarily from *Amanita phalloides* (Matinkhoo et al. 2018). Both phallotoxins and amatoxins are produced on the ribosome (Hallen et al. 2007), unlike many fungal chemical products, which are produced with nonribosomal peptide synthetases (NRPSs) (Bushley and Turgeon 2010). Though the genes encoding the cyclic peptide precursors are known (Hallen et al. 2007), the exact enzyme(s) responsible for the hydroxylation and epimerization processes remain elusive (Luo et al. 2018). In 2018, alpha-amanitin was synthesized chemically (Matinkhoo et al. 2018), but the process has yet to be scaled for mass production.

Before the effects of such compounds can be studied, the compounds must first be extracted and isolated. Procedures for extracting amatoxins from mushrooms have been evolving since the middle of the last century (Wieland et al. 1954). All of the amatoxin containing *Amanita* species are ectomycorrhizal, forming an obligate mutualism with trees, and thus can not be grown in the lab (Smith and Read 2008). Mushroom fruiting is often seasonal, so the fruiting bodies must be collected over a relatively short period. A common protocol is to dry the mushrooms to prevent spoilage, allowing more time for extraction than work with fresh mushrooms, and then to grind the samples to a fine powder (Sgambelluri et al. 2014; Garcia et al. 2015). Most such studies have been performed on *Amanita phalloides*, due its widespread availability and ample size. Previous extraction methods were often developed for preparing samples for instrumental detection. Most of these methods used solvent-based liquid extraction and required chromatography to ensure sufficient separation of other potentially interfering compounds within the sample, prior to detection by UV or mass spectrometry (MS).

Compared to MS, immunochemical (antibody-based) detection methods are less prone to interfering compounds, but are not compatible with high amounts of solvents. Recently we developed immunochemical detection methods (ELISA and LFIA) for amatoxins (Bever et al. 2019; Bever et al. 2020). During this process, we adjusted the extraction procedure to be compatible for antibody-based detection, which meant removing the use of the organic solvent, methanol. For the purposes of making a rapid field portable detection method, we also identified that sample maceration was not needed to extract the amatoxins and that the incubation time for extraction could be accomplished in 1 minute.

In this current work, we hypothesized that these same extraction adjustments–minimal tissue maceration and reduced incubation time–could be transferable to instrumental (e.g., UV or mass spectrometry) detection methods. To test the need for sample maceration, we utilized three different techniques: 1) traditional mortar and pestle, 2) a similarly disruptive method of bead beating, and 3) no grinding, but rather hand shaking dried mushroom tissue in the extraction buffer. In addition, we performed the solvent extraction step at varying times to observe if more time allows for more toxin to be removed from the tissue. Lastly, we utilized two comparable solvent evaporation methods (rotovap or speedvac) to establish if multiple samples could be processed simultaneously, thus improving sample throughput.

2.3. Materials and methods

2.3.1. Extraction protocols

A single large dried *Amanita phalloides* mushroom was selected for analysis. The mushroom was collected from Point Reyes National Seashore in 2017, under permit #PORE-2017-SCI-0054. Prior to extraction, the mushroom was re-dried at 113° F until it reached a constant weight.

The mushroom cap was radially divided into 16 pieces: it was first cut into four quadrants, then each quarter was further divided into four pieces. For the bead beating and hand shaken samples, pieces of dried mushroom were added to each vial, with a mass between 0.9 and 1.1 g. For the remaining samples, the rest of the mushroom cap was frozen with liquid nitrogen and ground with a mortar and pestle. Then, 0.9-1.1 g of mushroom powder was weighed into each 15 ml Falcon tube.

Bead beating was performed in 2.0 mL polypropylene screw cap vials (BioSpec Products, Inc., Bartlesville, OK, USA). Approximately 50 mg of 1.3 mm chrome steel beads (BioSpec Products) were used and the sample was shaken on a Mini-Beadbeater 24 (BioSpec Products) for 2 minutes to pulverize the dry sample.

To each sample type, we then added 1 ml of extraction solution (80% methanol: 10% .01M HCL: 10% ddH_20) (Walton 2018) per 0.02 g of mushroom tissue. Samples were incubated for various incubation times; 1, 5, 10, 30 and 60 minutes. Samples that were only incubated for one minute were completed at room temperature. The samples that were incubated for 5, 10, 30 and 60 minutes were placed in a 30°C incubator and gently rocked for the allotted time.

Next, samples were centrifuged for 10 minutes at 4000 g, except for the hand shaken samples; for these, the volume was transferred to the next container with an

automatic pipetman. The samples were then rotary evaporated under vacuum with either a speedvac (temperature 45°C, Vacuum: 5) or rotovap (bath temperature 40°C). Rotovapped samples were evaporated in a 100 mL round-bottom flask.

Once the sample was completely dried, it was resuspended in 100 ul of LCMS-grade water per ml of extraction solvent used. The final samples were diluted (15 ul of sample, 50 ul of water) before being run on HPLC. Each treatment was repeated in duplicate.

2.3.2. HPLC

Extracts were analyzed on an Agilent 1200 series HPLC coupled to a UV detector. Compounds were separated over a Phenomenex Kinetex XB-C18 column (100 × 3 mm, 100 Å, 2.6 µm particle size) column held at 50^{o} C using a gradient method with a mobile phase consisting of 20 mM ammonium acetate pH 5 (solvent A) and acetonitrile (solvent B). The gradient was as follows: 0-4 min. 6% B, 4-5 min. 6-15% B, 5-10 min. 15-18% B, 10-12 min. 18-60% B, 12-13 min. 60% B, 13-13.5 min. 60-6% B, 13.5-16 min. 6% B. Compounds were detected using a UV detector programmed to monitor wavelengths of 295 nm and 305 nm. Peaks were integrated in Agilent OpenLAB software. Alpha-, beta- and gamma-amanitin were quantified using the UV signal at 305 nm.

Dilutions (ranging from 12.5 - 250 ug/mL) of alpha- (Sigma, ≥90% purity), beta- (Sigma, ≥95% purity) and gamma-amanitin (≥90%, Enzo Life Sciences, Farmingdale, NY, USA) standards in LCMS-grade water were assessed in order to generate estimates of each toxin concentration. Linear regression analysis was performed using MassHunter software.

2.3.3. Data Analysis

All statistical analyses were carried out using Python Scipy. First, a test for normality was completed for each analyte. For those analytes with normally distributed data, t-tests were completed between each treatment type, while for non-normally distributed data sets, Mann-Whitney U tests were performed. The Bonferroni correction was applied to account for multiple comparisons. Comparisons were completed using the duplicate values from each evaporation type, between time points, and between the different methods used to macerate tissue samples.

2.4. Results

2.4.1. Overall toxin analysis

All samples in this study were analyzed using an HPLC-UV method. Liquid chromatography provided separation of the extracted components so that each compound could be detected independently. The retention times for alpha-, beta-, and gamma-amanitin were 3.821, 2.061, and 8.213 minutes, respectively. For statistical comparison between sample preparation methods, we utilized raw data (peak area). In

every experimental condition tested, all three target compounds (a-AMA, b-AMA, g-AMA) were detected.

First a test of normality was performed on the data, which found that only the data for beta-amanitin was normally distributed. Kruskal Wallis tests were then run on the data from all three toxins, which found that extraction method was significant ($p \leqslant 0.05$ for each toxin), but found no significance due to drying method ($p > 0.05$ or time ($p > 0.05$) for any toxin.

Data from each toxin were then analyzed independently. For alpha-amanitin and gamma-amanitin, Mann-Whitney U tests were performed, but no differences were statistically significant. Because the beta-amanitin data were normally distributed, we investigated the data with t-tests followed by a Bonferroni correction for multiple comparisons.

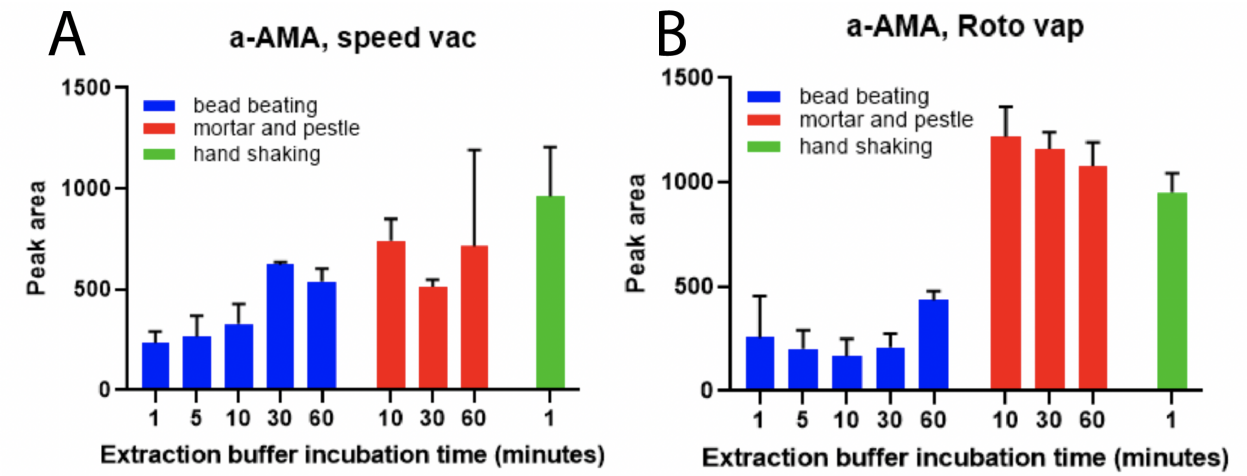

Figure 2-1 Extraction of Alpha-amanitin. Concentrations of Alpha-amanitin from mushroom extracted samples evaporated by (A) speed vac or (B) roto vap. Values are means +/- standard deviation (n=2). There were no statistically significant differences between any treatments.

2.4.2. Beta-amanitin

For the samples that were dried with speedvac, hand shaking was not significantly different than any of the bead-beat samples (two-tailed t-tests, $p > 0.05$). There were also no statistical differences between hand shaking and mortar and pestle at any of the time points (two-tailed t-tests, $p > .05$).

For the samples that were dried with rotovap, hand shaking yielded significantly more toxin than bead beat samples incubated for every time point except for 1 minute (two-tailed t-tests, $p < .05$). There were no significant differences between hand shaking and mortar and pestle at any time points.

Comparing rotovap and speedvac, there were several significant differences between samples that were bead beat (two-tailed t-tests, $p < .05$). Beadbeat samples that were incubated for 10 minutes and rotovapped were significantly different from samples that were dried with speed vac and incubated for 30 minutes ($p = .03$) and 60 minutes ($p = .03$). Similarly, beadbeat samples that were incubated for 30 minutes and

rotovapped were significantly different from samples that were dried with speed vac and incubated for 30 minutes ($p < .05$) and 60 minutes ($p < .05$). There was no statistical difference between samples that were hand-shaken (two-tailed t-test, $p = 1.93$). For samples that were ground with mortar and pestle, there was a significant difference between 30 minute rotovap and 30 minute speedvac (two-tailed t-test, $p = .03$).

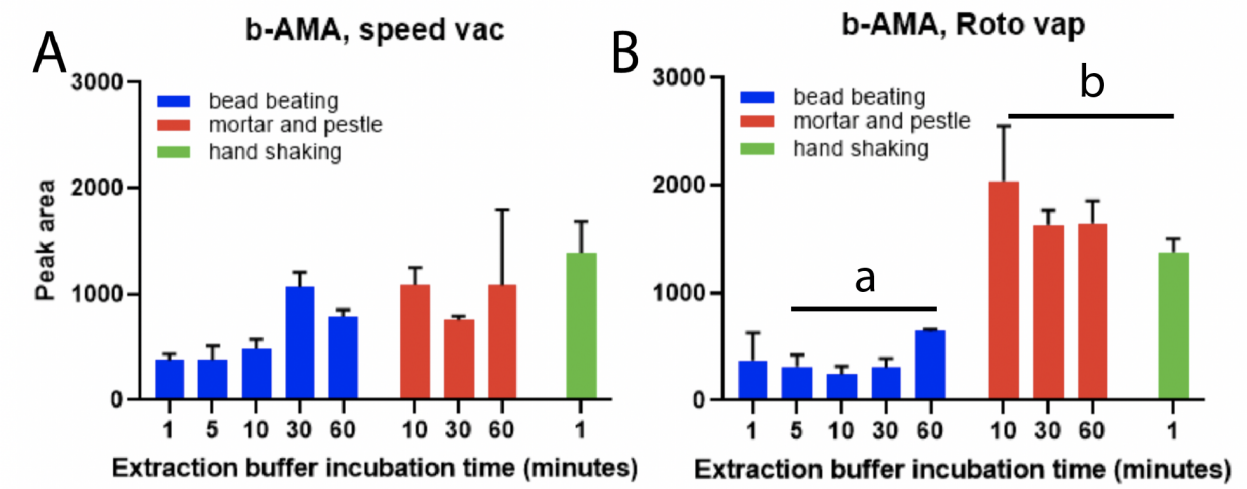

Figure 2-2 Extraction of Beta-amanitin. Concentrations of Beta-amanitin from mushroom extracted samples evaporated by (A) speedvac or (B) rotovap. Values are means +/- standard deviation (n=2).

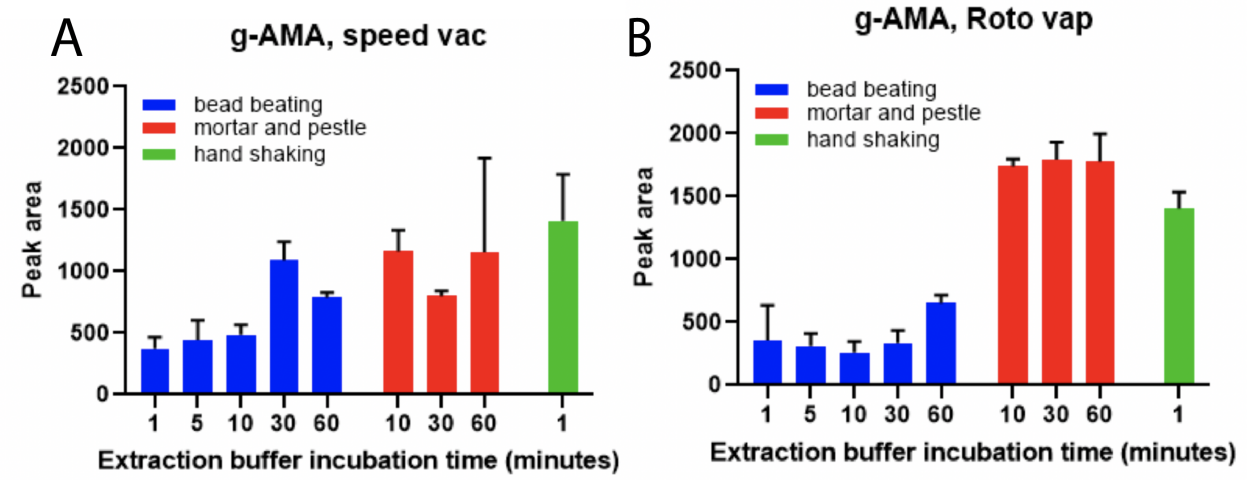

Figure 2-3 Extraction of Gamma-amanitin. Concentrations of Gamma-amanitin in mushroom extracted samples evaporated by (A) speedvac or (B) rotovap. Values are means +/- standard deviation (n=2).

2.5. Discussion

In this study, we extracted amatoxins using modified sample preparation methods. In many cases, previous studies focused on the development of analytical chemistry techniques, and not the wet lab steps leading to chemical analysis (Tanahashi et al. 2010; Nomura et al. 2012; Yoshioka et al. 2014; Zhang et al. 2016). We observed that in many of the previously reported extraction methods, incubation time with the extraction solvent had been reduced from 24 hours down to less than an hour. Early protocols incubated for several days (Wieland and Wieland 1959), and then later for 24 hours or overnight (Mcknight et al. 2010; Clarke et al. 2012; Kaya et al. 2013). Only a handful of studies incubated for as short a period as an hour (Stijve and Seeger 1979; Jansson et al. 2012; Sgambelluri et al. 2014; Garcia et al. 2015). The shortest incubation step we could find in the literature was 10 minutes (Ahmed et al. 2010). In this study, we provide more evidence that reduced incubation times, down to as little as 1 minute, are sufficient to achieve toxin extraction from dried mushroom tissues.

At the outset of each extraction, most methods are performed on ground or macerated dried mushroom tissue (Sgambelluri et al. 2014; Garcia et al. 2015). This is both time-consuming and can generate airborne dust, which could increase exposure of the researcher and their environment to toxic materials. To reduce airborne dust production, we examined if bead beating, an equally destructive method for macerating tissue, which is self-contained within a tube, could be a suitable alternative. Our results indicate that the toxin is extracted through bead beating, although for beta-amanitin samples that were rotovapped, the amount of toxin extracted from bead beaten samples was considerably less than the amount of toxin extracted from hand-shaking the sample (**Figures 1-3**). With bead beating, the higher variability and recovery loss was likely due to the volume of fluid trapped on and among the beads, thus reducing overall recovery. Recovery could be improved by washing the beads. However, the disruption to the cell wall does not seem needed, given how well the hand-shaken samples were extracted.

As another alternative to reduce dust production, we examined the feasibility of extracting toxins by simply dropping a small piece of dried tissue in a tube and hand-shaking the tissue in extraction solvent. Our results indicate that hand shaking a piece of mushroom for a minute can yield as much toxin as a more elaborate protocol involving grinding the tissue with liquid nitrogen followed by an hour-long incubation step (**Figures 1-3**). The only potential drawback of hand-shaking is that centrifuging is not as effective with mushroom pieces as it is with mushroom powder, and there is risk of small tissue transfer after the incubation step. This could possibly be reduced by performing a simple filtration of the extract.

This study is, to our knowledge, the first comparison of rotovap and speedvac on recovery of mushroom toxins, and perhaps the first such comparison of any biological compound. Some early studies on amatoxin extraction did not specify what type of equipment was used to evaporate solvent, instead simply stated that the solvent was evaporated under vacuum (Faulstich et al. 1973; Stijve and Seeger 1979). Our results indicate that, for alpha-amanitin and gamma-amanitin, evaporating multiple samples simultaneously with a speed-vac yielded as much toxin as evaporating samples one at a time with a rotovap, but the same was not always true for beta-amanitin (**Figures 1-3**). If the goal is to maximize the amount of amatoxin extracted, then rotovap may be more

appropriate. We think that, for beta-amanitin, the yield with rotovap is higher than speedvac because, with the speedvac and running multiple samples simultaneously, the extract can sometimes over-dry to the walls of the container, and fail to re-dissolve. This over-drying may occur with these mushroom toxins as well as many other biological compounds, and warrants further investigation. Future work could also consider comparing rotovap and speedvac to evaporating solvent under a liquid nitrogen stream (Clarke et al. 2012).

Our results allow us to theorize about the cellular nature of the toxin within the mushroom. Because hand shaking was always as efficient as mortar and pestle at extracting amatoxins, we can surmise that amatoxins are not deeply embedded in cell walls or compartments, and thus do not need extensive pulverization for extraction. This observation that the amatoxins are not deeply embedded allows us to postulate about the ecological role of amatoxins. Such readily released toxins may be more accessible to fungivores such as *Amanita*-associated *Drosophila* species (Greenleaf et al. 1979; Stump et al. 2011; Mitchell et al. 2017), and thus act as a potent deterrent against mycophagy.

The ease of extraction can also help to explain the bioavailability of the amatoxin. For animals, the toxicity of amatoxins is directly related to the ability of the species in question to absorb the toxins in the gut (Wieland and Faulstich 1978). As recently as this last decade, it has been erroneously stated that the toxins were not water soluble (Allen et al. 2012), and this idea has perpetuated throughout some mycologist communities. Our work suggests that even the commonly practiced method of 'taste testing' a small piece of mushroom would yield toxin exposure to a person and thus is not recommended when one suspects the mushroom to be a species that contains amatoxins. Perhaps the toxin is produced in such relative excess because it can readily leave the mushroom with water, such as with precipitation or fog drip. Once in the soil, amatoxins could function not only as a defense chemical but perhaps also an offense chemical, inhibiting nearby fungi and other eukaryotic soil microbes.

In conclusion, we present an extraction protocol that saves time, reduces equipment contamination, and minimizes risk to the researcher. Using liquid nitrogen and grinding the mushroom to a fine powder poses potential harm to the researcher and potentially contaminates tools and the surrounding environment. In contrast, hand-shaking a piece of mushroom reduces occupational hazards for the scientists, allowing them to reduce both specimen handling time and destructive manipulation methods. Toxicologists and officials should be aware of potential nefarious acts to endanger pets (dogs and cats) and humans, considering the ease of toxin extraction with minimal equipment and technical experience, and that this species is still undergoing a range expansion (Wolfe and Pringle 2012). Furthermore, the impact of this faster, safer method may help produce these important toxins faster, for both research and medical use.

2.6. Acknowledgements

The authors wish to thank Jacob Baker, Sean Brown, and Stephen Shen for their assistance with protocol development. Sincere gratitude to the Traxler lab for use of

their speedvac and rotovap. Thank you to the Bruns and Taylor labs for feedback on an early version of the manuscript. Finally, thank you to Jonathan Walton for the gift of his original extraction protocol, as well as his enthusiasm and insight.

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Chapter 3. Amatoxin and phallotoxin levels of *Amanita phalloides* in California across multiple scales: within a mushroom, between sites, and over time

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3.1. Abstract

The death cap mushroom, *Amanita phalloides*, wields a defensive chemical arsenal of both amatoxins and phallotoxins. While the toxin levels of *A. phalloides* have been described from regions in its native range of Europe, little work has examined toxin levels where it has been introduced and become invasive, such as California, USA.

Here, we use High Performance Liquid Chromatography (HPLC) and Liquid Chromatography High Resolution Mass Spectroscopy (LC-HRMS) to measure toxin levels of the two primary amatoxins and the two primary phallotoxins to ask the following questions of *Amanita phalloides* mushrooms collected from native California forest: 1) Does toxin concentration vary within a single pileus? 2) Do toxin concentrations vary across the different organs of an individual mushroom (pileus, stipe and volva)? 3) How much do toxin concentrations differ between sites in a native forest? And finally, 4) Does toxin concentration differ between years at the same site?

We found that, for alpha-amanitin and phalloidin, toxin levels hardly vary within a pileus, while beta-amanitin and phallacidin showed considerably more variation. When we examine mushrooms from multiple sites, we find strikingly little variation between the different mushroom compartments, but for each toxin examined, the volva generally contained the most toxin. This trend is different from patterns of mushrooms in Europe, where amatoxin levels are highest in the pileus, while phallotoxin levels are higher in the volva. Temporally, at each site, toxin levels generally increased from one year to the next. Levels of alpha-amanitin in California are on the high end of published European values, indicating that either this invasive species underwent a genetic bottleneck from an area of Europe with high toxin levels, or *A. phalloides* may be under active selection to increase toxin levels in its introduced range.

3.2. Introduction

The death cap mushroom, *Amanita phalloides* (**Figure 1**), is often reported as the world's deadliest mushroom, causing more deaths than any other fungus species (Enjalbert et al. 2002). Its toxins are so potent that consuming half a pileus can kill an adult human (Clarke et al. 2012). It is estimated that between 200 and 250 people are poisoned by mushrooms each year globally (Govorushko et al. 2019), and of these poisonings, 90% of fatalities are attributed to species containing amatoxins (H. Bresinsky and Besl 1989; Mengs et al. 2012).

Amanita phalloides possesses two families of ribosomally synthesized cyclic peptides that cause different effects on the cell: the amatoxins and the phallotoxins **(Figure 2)**. The phallotoxins are cyclic heptapeptides that prevent the polymerization of F-actin in all eukaryotes (Wieland and Govindan 1974; Vetter 1998), as well as actin-like proteins in bacteria, e.g. *Anabaenea cylindrica* (Guerrero-Barrera et al. 1996; Guljamow et al. 2012). This binding inhibits cell motility and muscular contraction, altering cell shape. The phallotoxins, however, are not absorbed in human stomachs (Wieland and Faulstich 1991), and are not thought to play a role in human poisonings, unlike the amatoxins. The amatoxins are a related family of cyclic octapeptides that bind RNA polymerase II in eukaryotes, halting transcription and causing cell death (Brodner and Wieland 1976; Gong et al. 2004; Carter and Drouin 2009). In humans, the amatoxins accumulate in the kidneys (Mengs et al. 2012) and can enter the liver via the organic anion transporting polypeptides (OATP) receptors (Letschert et al. 2006).

Though the role these toxins play in human health has been well studied, the literature remains undecided on what role, if any, the toxins play ecologically. Some mushroom-affiliated invertebrates are resistant to amatoxins, such as *Drosophila* species (Begun and Whitley 2000; Stump et al. 2011; Mitchell et al. 2014) and the nematode *Caenorhabditis elegans* (Sanford et al. 1983). Toxin production is not constant, geographically or over a season, further suggesting an ecological role of toxins. Sporocarps of *A. phalloides* have been found to produce different levels of toxins between sites (Garcia et al. 2015), and even within a site (Enjalbert et al. 1996). *Amanita phalloides* also exhibits toxin variation with respect to the mushroom compartment; amatoxins are concentrated in the pileus, while phallotoxins are concentrated in the stipe and volva (Enjalbert et al. 1996). However, most studies on toxin levels in *Amanita phalloides* have been performed on samples from Europe, in the native range of this fungus.

Once found only in Europe, *A. phalloides* was accidentally introduced around the world with European host tree seedlings during the first half of the last century (Vellinga et al. 2009; Pringle et al. 2009; Wolfe et al. 2010). *Amanita phalloides* is ectomycorrhizal, forming an obligate symbiosis with tree roots, wherein the plant trades photosynthate for nitrogen, phosphorus, trace minerals, and water (Smith and Read 2008; Wolfe and Pringle 2012). When European plants were brought to new locations, such as California, USA, the plants were brought with intact root systems and European soil (Pringle et al. 2009). *Amanita phalloides* was apparently introduced with these plants and then spread from its initial introduction points, by wind-dispersed spores (Golan et al. 2019). At the northern end of its West coast range, *A. phalloides* is still spreading into British Columbia, where it now associates with native trees (Berch et al 2016). *Amanita phalloides* has undergone a similar host shift in California, where the

fungus associates with the California live oak, *Quercus agrifolia* (Wolfe and Pringle 2012). Thus in California, *A. phalloides* is not just introduced, but invasive (Dickie et al. 2016).

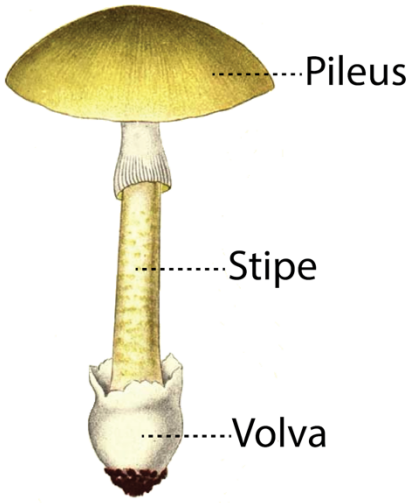

Figure 3-1 *Amanita* physiology. The three main compartments of a mature mushroom of *Amanita phalloides*: pileus (cap) stipe (stem) and volva (bulb).

While a considerable amount of work has examined toxin levels of *A. phalloides* in its native range, few studies have quantified toxin levels in California, where *A. phalloides* is invasive (Ammirati et al. 1977; Sgambelluri et al. 2014). No studies have examined toxin level variation within a single mushroom cap, to determine how representative a piece of the pileus is of the whole pileus. Nor has anyone investigated differences across the different compartments of an individual mushroom, which may give clues to which compartments are most under selection. Lastly, no studies have established how toxin levels may differ over time or across sites in California, especially in an ecological context. Here, we study mushrooms from sites in Point Reyes National Seashore, which have been producing mushrooms for at least five years prior to the current sampling (Wolfe et al 2011). We use High-Performance Liquid Chromatography (HPLC) and Liquid Chromatography High Resolution Mass Spectroscopy (LC-HRMS) to measure toxin levels of the two primary amatoxins (**Figure 1a, 1b**) and the two primary phallotoxins (**Figure 1c, 2d**) to ask the following questions of *Amanita phalloides* mushrooms collected from native California forest:

1) Does toxin concentration vary within a single pileus?

2) Does toxin concentration vary across the different compartments of an individual mushroom (pileus, stipe and volva)?

3) How much do toxin concentrations differ between established sites in a native forest?

4) Does toxin concentration differ between years at the same site?

5) How do toxin levels in California mushrooms compare to published toxin values of European mushrooms?

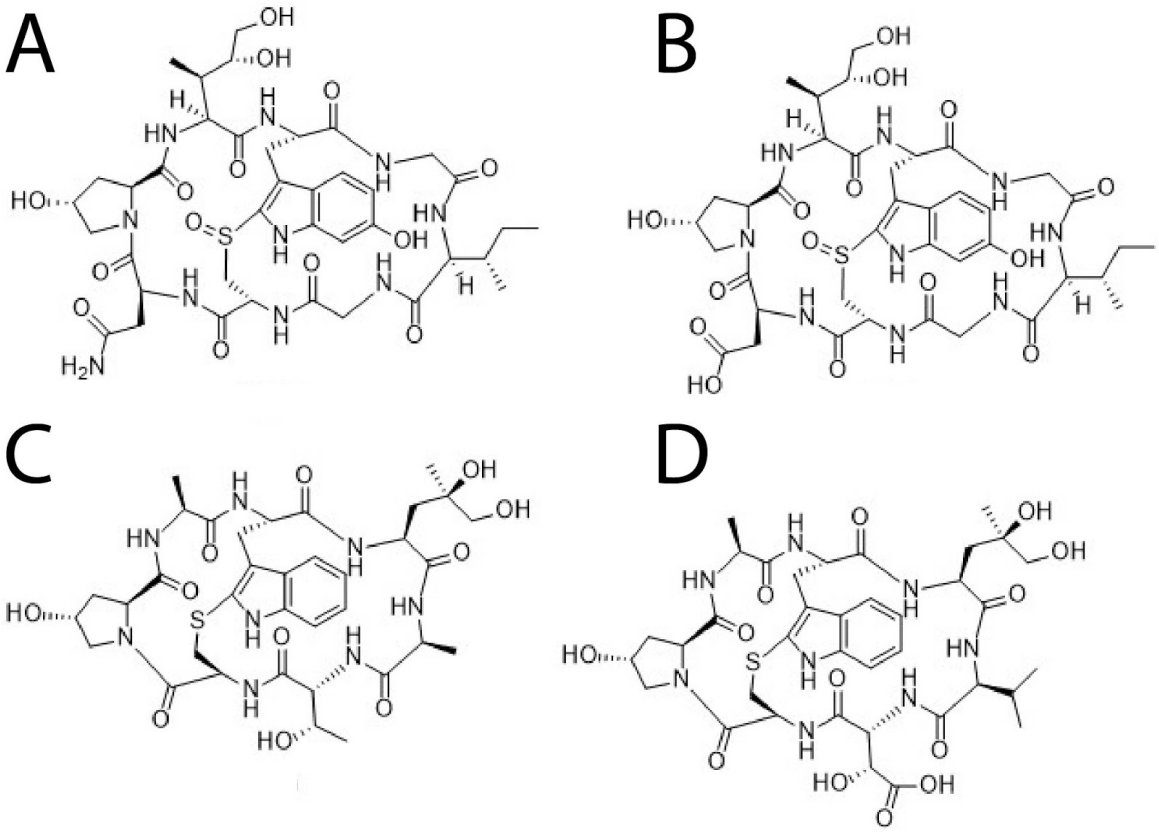

Figure 3-2 Primary amatoxins and phallotoxins of *Amanita phalloides*. A) Alpha-amanitin B) Beta-amanitin C) Phalloidin D) Phallacidin.

3.1. Materials and methods

3.1.1. Collection of Mushrooms

All mushrooms were collected from Point Reyes National Seashore under permit #PORE-2017-SCI-0054. Mushrooms were collected during autumn of the years 2014, 2015, 2016, and 2017, and the timing of collection was approximately mid-season each year (**Table 1)**.

Sites

We define a site as a group of mushrooms occurring within an area no larger than 75m by 75m (Golan et al. 2019). The Drake sites were delineated arbitrarily by walking either across Limantour road, or walking at least 75m from the first site, before collecting and naming a distinct site (Wolfe et al. 2010; Golan et al. 2019).

Table 3-1 *Amanita phalloides* collection sites.

Sites used in this study, their GPS coordinates, the dates mushrooms were collected each year, and the number of unique mushrooms (n) involved in this study.

	Latitude	**Longitude**	**2014**	**2015**	**2016**
Drake 2	38.054785 N	122.833232 W	Nov 17 (n = 44)	Dec 13, 14 (n = 11)	Nov 12
Drake 3	38.055212 N	122.834134 W	-	Dec 13, 14 (n = 3)	Nov 12
Drake 3.5	38.054845 N	122.836342 W	-	Dec 13, 14 (n = 4)	Nov 12
Pet	38.03237 N	122.4839 W	-	Dec 13, 14 (n = 7)	Nov 12 (n = 3)

3.1.2. Mushroom processing:

Collected mushrooms were all mature with pileus fully open, and were dried for 48-72 hours at 113° F until constant weight. Dried mushrooms were stored in Ziplock bags at ambient temperature.

3.1.3. Variation within a mushroom

To examine variation within a single individual mushroom, a large, mature mushroom collected in 2016 from “Pet” (Accession 10917) was selected. The pileus was divided radially into 8 pieces. The stipe was divided into thirds: lower stipe, middle stipe, and top stipe. The volva was divided in half, such that each piece contained both lower and upper portions of the volva.

Next we asked if there was variation in toxin levels between the edges of the pileus compared to the center. To do this, a 10 x 10 cm grid was placed over a mushroom collected from Drake 2 in 2017 (Accession 10965) (**Figure 4A, 4B**). The pileus was divided into 16 pieces as evenly as possible (**Figure 4C**), and the pieces were assigned numbers from left to right, one row at a time. Four pieces (6, 7, 10, 11) were considered “center,” while the remaining 12 pieces were “edge.”

To examine toxin level variation across the mushroom compartment and field site, we selected at least 3 mushrooms from each site for analysis. Mushroom pilei were divided radially into 8 pieces. Mushroom stipes were split down the middle sagitally. The lower half of each length was designated “lower stipe,” while the upper half was “upper stipe.” The volvas were divided as for Accession 10965.

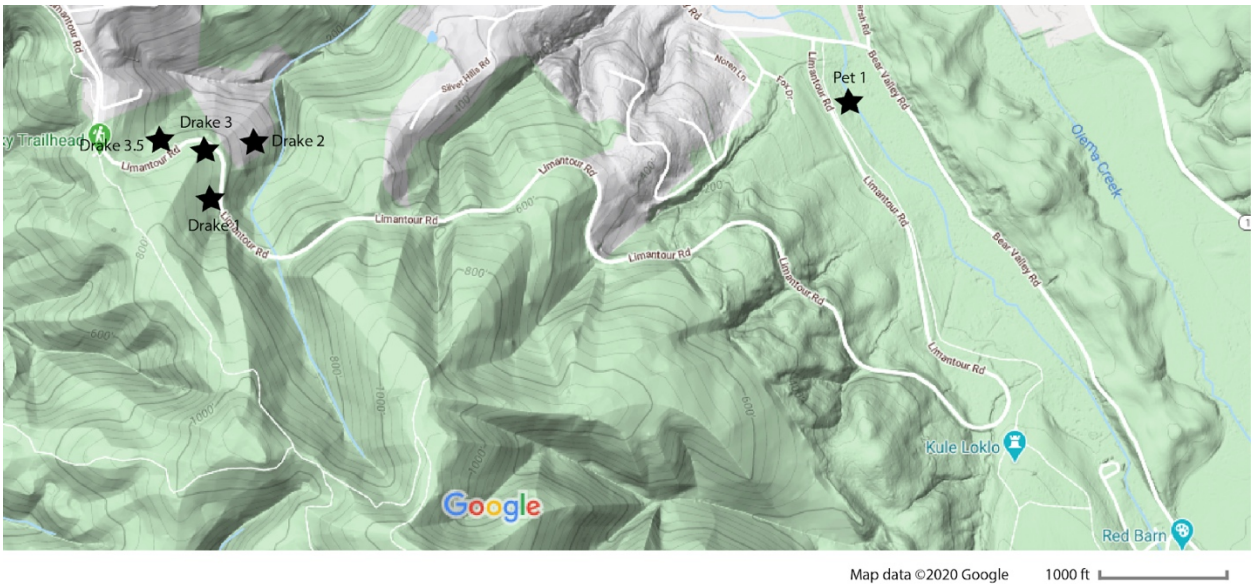

Figure 3-3 Location of collection sites. Topological map of the region of Pt Reyes National Seashore containing the collection sites. Map data courtesy of Google Maps**.**

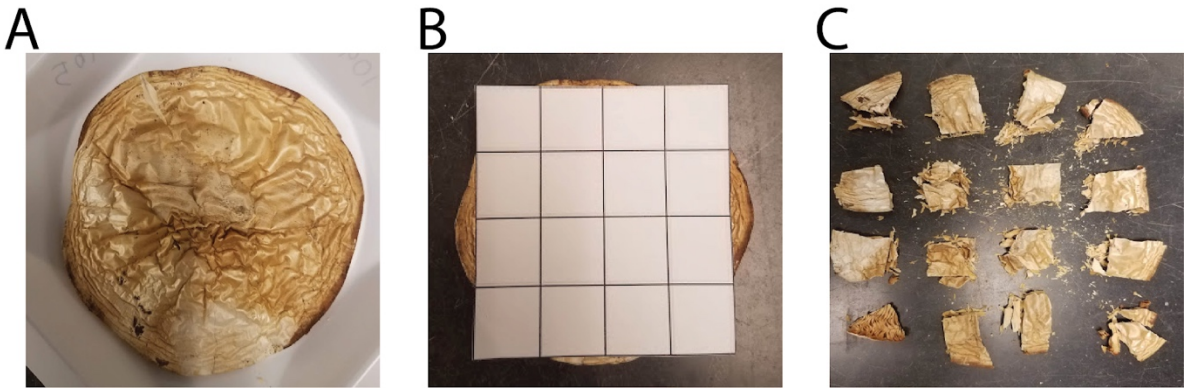

Figure 3-4 Division of mushroom number 10965. A) Pileus after re-drying. B) A 4 x 4 inch grid overlaid on the mushroom C) The 16 pieces.

3.1.4. Extraction procedure

Prior to extraction, each mushroom was re-dried at 113° F until it reached a constant weight. Mushrooms were processed as described previously (Adams 2020). Briefly, a piece of mushroom was frozen with liquid nitrogen and ground with a mortar and pestle. Then, 0.9-1.1 g of mushroom powder was weighed into a 15 ml Falcon tube. To each Falcon tube, 1 ml of extraction solution (80% methanol: 10% .01M HCL: 10% ddH_20) (Walton 2018) was added per 0.02 g of mushroom tissue. Samples were incubated for 60 minutes in a 30°C incubator and gently rocked for 1 hour. Next, samples were centrifuged for 10 minutes at 4000 g. The supernatant was transferred to

a 100 mL round-bottom flask, and rotary evaporated under vacuum with a rotovap (bath temperature 40°C).

Once the sample was completely dried, it was resuspended in 100 ul of LCMS-grade water per ml of extraction solvent used. The final samples were diluted (15 ul of sample, 50 ul of water).

3.1.5. Liquid Chromatography High Resolution Mass Spectrometry (LC-HRMS)

LC-HRMS analysis was performed using an Agilent Technologies 6510 Accurate-Mass QTOF LC-MS instrument. Chromatographic separation was achieved using an Agilent 1200 series HPLC and a Phenomenex Kinetex XB-C18 column (100 × 3 mm, 100 Å, 2.6 µm particle size) using a linear gradient of 10-40% acetonitrile in water with 0.1% formic acid over 10 minutes. Compounds were quantified using Agilent MassHunter Quantitative Analysis software. Purified standards were used to generate standard curves.

3.1.6. High Performance Liquid Chromatography (HPLC)

Extracts were analyzed on an Agilent 1200 series HPLC coupled to a UV detector. Compounds were separated over a Phenomenex Kinetex XB-C18 column (100 × 3 mm, 100 Å, 2.6 µm particle size) column held at 50^{o} C using a gradient method with a mobile phase consisting of 20 mM ammonium acetate pH 5 (solvent A) and acetonitrile (solvent B). The gradient was as follows: 0-4 min. 6% B, 4-5 min. 6-15% B, 5-10 min. 15-18% B, 10-12 min. 18-60% B, 12-13 min. 60% B, 13-13.5 min. 60-6% B, 13.5-16 min. 6% B. Compounds were detected using a UV detector programmed to monitor wavelengths of 295 nm and 305 nm. Peaks were integrated in Agilent OpenLAB software and quantified relative to a standard curve. Alpha-, beta- and gamma-amanitin were quantified using the UV signal at 305 nm.

3.1.7. Synthesizing published values for European specimens of *Amanita phalloides*

Toxin concentrations were taken from existing literature. Studies were included if they reported amatoxin concentrations using either Thin Layer Chromatography (TLC), HPLC, or LC-MS to quantify toxin concentrations. If multiple sites were sampled, values were averaged across sites. When mushroom pileus was subdivided into lammellae (gills) and pileus (e.g. (Enjalbert et al. 1996)), lamellae and pileus values were averaged.

3.2. Results

3.2.1. Toxin variation within a single mushroom

We first looked at variation in a mushroom, to determine how representative a fraction of the pileus or stipe is of the whole compartment. For individual 10917, the average toxin levels varied considerably by mushroom compartment (**Figure 5**). The average level of alpha-amanitin in the pileus was 0.96 +/- 0.11 mg/g dry mushroom

weight; in the stipe it was 0.39 +/- .03 mg/g; and in the volva the average was 0.37 mg/g. There was only one replicate for the volva, so no standard deviation could be calculated for that compartment. Examining beta-amanitin levels, the average level of toxin in the pileus was 1.44 +/- 0.47 mg/g; in the stipe the level was 0.40 +/- 0.07 mg/g; and the average level in the volva was 0.34 mg/g. For phallacidin, the average level of toxin in the pileus was 0.98 +/- .47 mg/g; in the stipe, the average was 0.42 +/- .22 mg/g; and the average for the volva was 0.28 mg/g. Lastly, for phalloidin, the average level in the pileus was 1.10 +/- .11 mg/g; in the stipe it was 0.97 +/- 0.29 mg/g; and in the volva the level was 1.50 mg/g. Based on these standard deviations, we deem alpha-amanitin and phalloidin to be more representative of the entire pileus than beta-amanitin and phallacidin, but all four toxins are generally evenly distributed.

To answer whether there were differences in toxin levels between the pileus and stipe of accession 10917, we performed Mann Whitney U tests. The difference was not significant for phalloidin ($p = .11$), but was significant for each of the other toxins: alpha-amanitin ($p = .001$), beta-amanitin ($p = .002$), and phallacidin ($p = .009$).

We then evaluated variation within the pileus of mushroom number 10965 to test whether there was a difference in toxin levels between pileus center and pileus edge. We did not find a statistically significant difference in toxin levels for any toxins (**Figure 6**) (Mann-Whitney U test: alpha-amanitin $p = 0.18$, beta-amanitin $p = 0.38$, phallacidin $p = 0.25$, phalloidin $p = 0.38$).

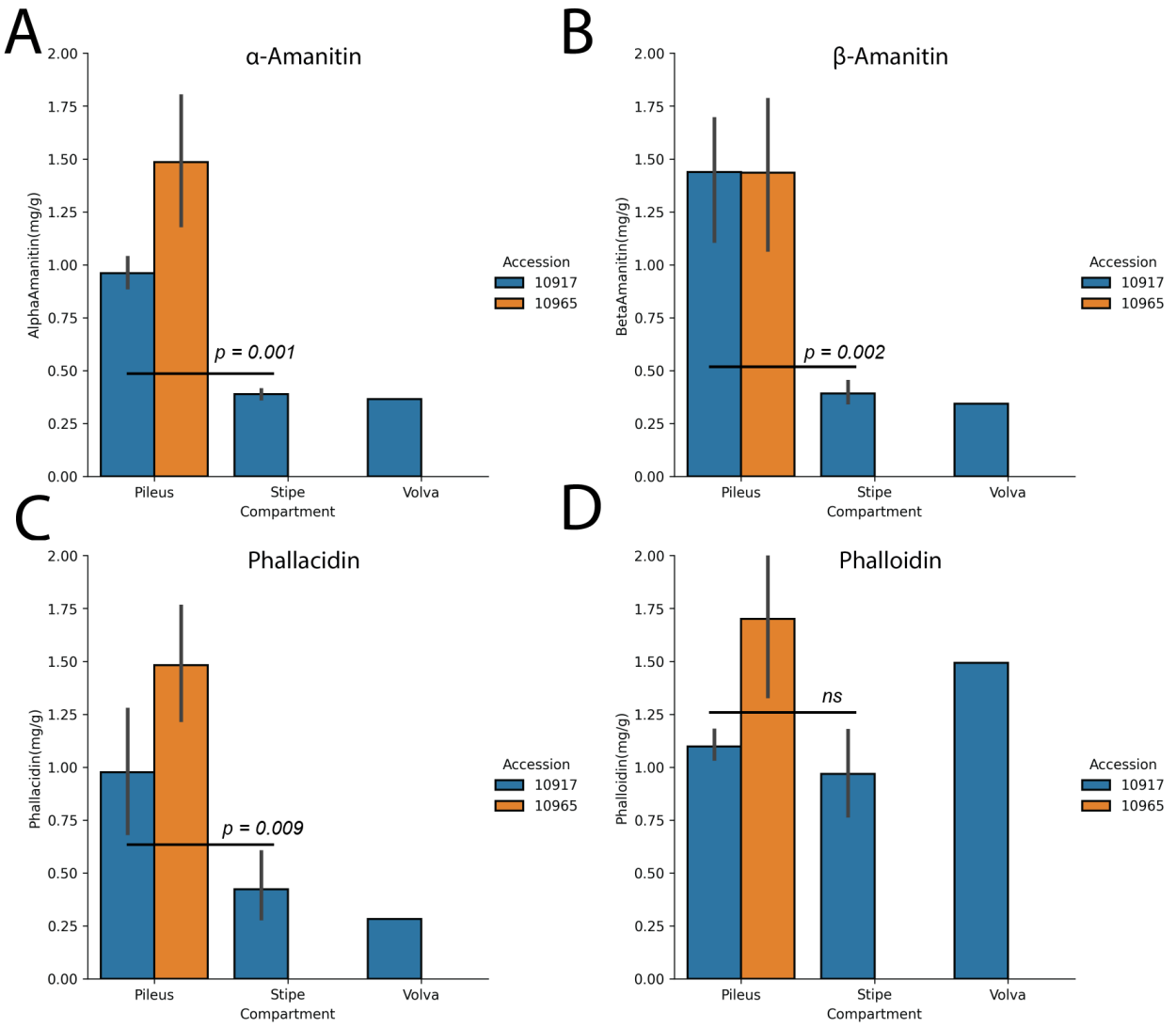

Figure 3-5 Intra-pileus toxin variation of mushrooms 10917 and 10965. A) Alpha-amanitin B) Beta-amanitin C) Phallacidin D) Phalloidin. Error bars show 95% confidence intervals.

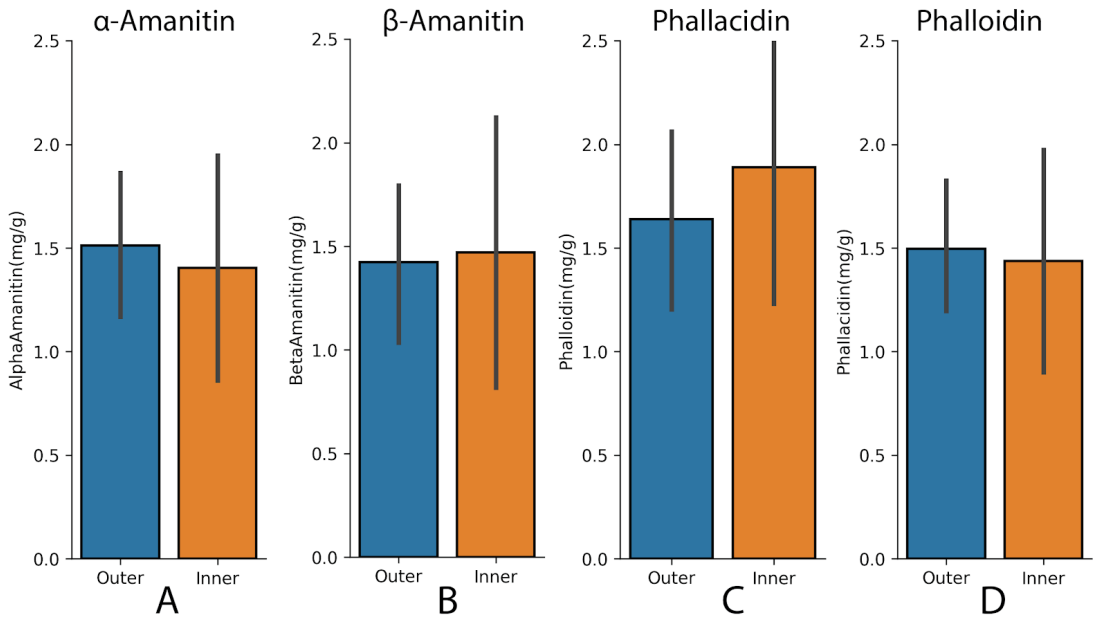

Figure 3-6 Intra-pileus toxin variation. A) alpha-amanitin. B) beta-amanitin. C) phallacidin D) phalloidin. Error bars represent 95% confidence intervals. There were no statistically significant differences in toxin levels between outer and inner pileus.

3.2.2. Variation in toxin level by compartment and site

To examine toxin level at an ecological scale, we next asked whether toxin levels varied by mushroom compartment and site. We first asked if there was variation in toxin levels across compartment and site, and performed a Kruskal-Wallis test. The results were statistically significant for every comparison (data not shown). To ask whether there were differences in toxin levels between sites, we then applied the Dunn's Multiple Comparison Test. We found significant differences for a number of comparisons (**Figure 7**).

For alpha amanitin, there were significant differences in pileus toxin levels between the site "Drake 3.5" and site "Drake 2" ($p = .04$), and between "Drake 3.5" and "Pet" ($p < .001$). There was also a significant difference between "Drake 3" and "Pet" ($p = .02$). Comparing alpha-amanitin across stipes, there was a significant difference between "Drake 3.5" and "Drake 2" ($p = .02$), and between "Drake 3.5" and "Pet" ($p < .0001$). There was also a significant difference between "Drake 2" and "Pet" ($p = .04$), and between "Drake 3" and "Pet" ($p = .003$). Comparing volva toxin levels, we found a significant difference between "Drake 3.5" and "Pet" ($p < .001$), and between "Drake 3" and "Pet" ($p = .01$).

Significance trends were similar for beta-amanitin. Comparing pileus levels, we found significant differences between "Drake 3.5" and "Drake 2" ($p = .01$), and between "Drake 3.5" and "Pet" ($p < .001$). There was also a significant difference between pileus levels of "Drake 3" and "Pet" ($p < .05$). Comparing stipe toxin levels, there were significant differences between "Drake 3.5" and "Drake 2" ($p = 0.02$) and between "Drake 3.5" and "Pet" ($p < 0.00001$). We also found a significant difference between

stipe levels of “Drake 2” and “Pet” ($p < 0.05$), and between “Drake 3” and “Pet” ($p < 0.003$). Comparing volva toxin levels, we found a significant difference between “Drake 3.5” and “Pet” ($p < 0.001$), and between “Drake 3” and “Pet” ($p = 0.01$).

Next we examined differences in phallacidin levels across compartment and site. We found a significant difference in pileus levels between “Drake 3.5” and “Pet” ($p < 0.001$), and between “Drake 3” and “Pet” ($p < 0.005$). As for stipe levels, there were significant differences between “Drake 3.5” and “Drake 2” ($p = 0.03$), and between “Drake 3.5” and “Pet” ($p = 0.03$). We also found a significant difference between “Drake 2” and “Drake 3” ($p < 0.04$), and between “Drake 3” and “Pet” ($p < 0.04$). There were no significant differences between volva levels of different sites, likely because phallacidin volva levels were low for most sites.

Toxin levels for phallacidin followed a similar pattern as phalloidin. Comparing across pilei, we found a significant difference between “Drake 3.5” and “Drake 2” ($p < .04$), and between “Drake 3.5” and “Pet” ($p < .0001$). There was also a significant difference between “Drake 3” and “Pet” ($p =0 .02$). Comparing stipe levels, we found a significant difference between “Drake 3.5” and “Pet” ($p < 0.00001$), and between “Drake 3” and “Pet” ($p < 0.003$). Lastly, comparing volva toxin levels, we found a significant difference between “Drake 3.5” and “Pet” ($p < 0.001$), and between “Drake 3” and “Pet” ($p < 0.02$).

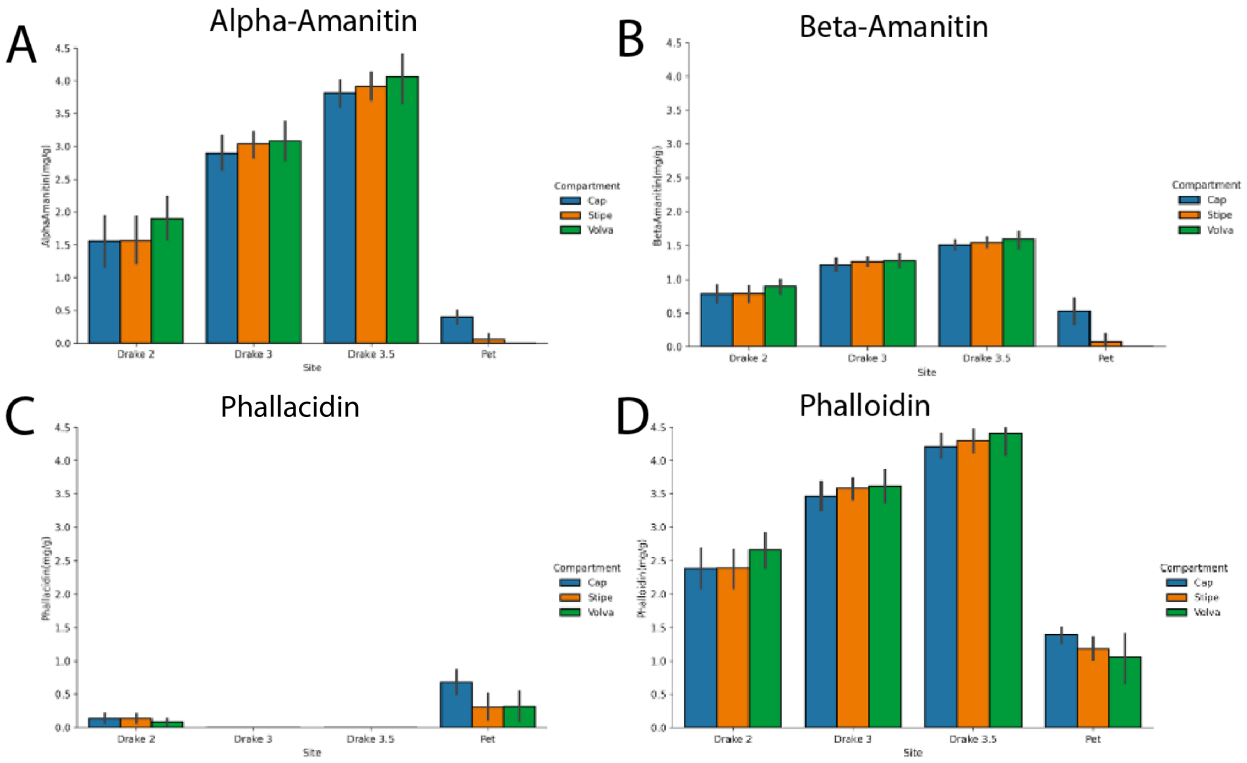

Figure 3-7 Intra-mushroom variation across sites and mushroom compartments. A) Variation in alpha-amanitin levels across mushroom compartments and sites. B) Variation

in beta-amanitin levels across mushroom compartments and sites. C) Variation in phallacidin levels across mushroom compartments and sites. D) Variation in phallacidin levels across mushroom compartments and sites. Error bars represent 95% confidence intervals.

3.2.3. Mushroom pileus toxin levels across site and year

Next we asked whether, at a particular site, there was a significant difference in toxin levels between years. To do this, we performed a Mann-Whitney U test. At the "Pet" site, there was no statistical difference in phalloidin levels ($p = 0.2$), but for the other toxins, there was significantly more toxin in 2016 than 2015: alpha-amanitin ($p < 0.005$), beta-amanitin ($p < 0.005$), and phallacidin ($p < 0.005$). At the site "Drake 2," there was significantly more toxin in 2015 compared to 2014 for the two amatoxins, alpha-amanitin ($p < 0.005$) and beta-amanitin ($p = 0.04$), and for phalloidin ($p < 0.005$). For phallacidin, there was significantly more toxin in 2014 compared to 2015 ($p < 0.005$).

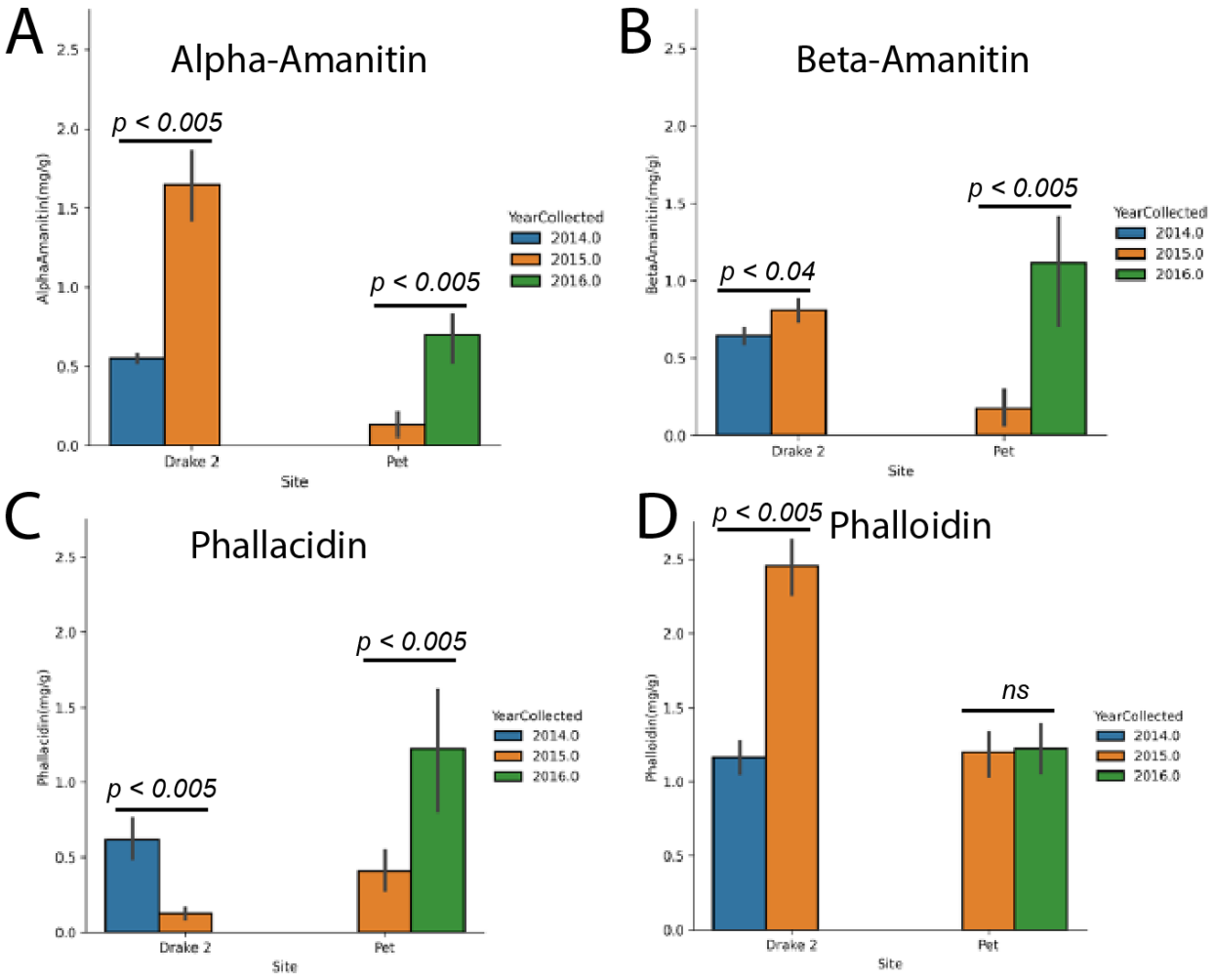

Figure 3-8 Intra-mushroom toxin variation within the pileus across sites and year. A) Variation in alpha-amanitin levels across mushroom via year and site. B) Variation in beta-amanitin levels across mushroom via year and site. C) Variation in phallacidin levels across mushroom via year and site. D) Variation in phallacidin levels across mushroom via year and site. Error bars represent 95% confidence intervals.

3.2.4. Comparing California toxin levels to published values of alpha-amanitin found in *European* samples

Previously published values of toxin concentrations of European specimens are presented in **Table 2**. In Europe, across several studies, pileus alpha-amanitin ranged from 0.123 mg/g to 2.95 (**Table 2**), while in California we found it ranged from 0.3 (Pet, 2015) to as high as 3.75 (Drake 3.5, 2015) (**Figure 7**). Similarly, within the stipe, concentrations of alpha-amanitin in European specimens ranged from 0.67 to 1.75 mg/g (**Table 2**), while in California the range was higher: 0.1 to 3.8 mg/g (**Figure 7**). However, the same trend was not true for the volva: concentrations in Europe ranged from 0.089 to 2.8 mg/g, while in California we found the levels were lower, from undetectable to about 1 mg/g.

Table 3-2 Concentration of alpha-amanitin from European *Amanita phalloides*.

Alpha-amanitin concentrations (mg/g) in the pileus of samples of *Amanita phalloides*. Hyphens indicate the value was not reported. *Wet weights reported.

	This Study	**Sgambelluri et al 2014**	**Andary et al 1977**	**Enjalbert et al 1996***	**Garcia et al 2015**	**Kaya 2013**	**Sgambelluri et al 2014**	**Stijve and Seeger 1979**
Pileus	1.88	0.88	2.65	0.123	0.784	2.95	1.33	2.3
Stipe	1.22		0.95	-	0.723	2.3	-	-
Volva	1.54		-	-	0.089	2.8	-	-
Region	California, USA	California, USA	France	France	Portugal	Turkey	Italy	Germany and Switzerland

3.3. Discussion

Here, we use HPLC and LC-HRMS to quantify toxin levels of the introduced fungus *Amanita phalloides* collected from native forest in California, USA at multiple scales. We first investigated toxin variation within an individual mushroom (**Figure 5**). For Accession 10917, we found significantly more toxin in the pileus compared to the stipe for three of the toxins (alpha-amanitin, beta-amanitin, and phallacidin), but not for phalloidin. For phalloidin, we found considerably more toxin in the volva than in the pileus and stipe. By dividing up a different mushroom in a grid pattern (**Figure 4**), we also examined whether the center of the cap contained more or less toxin than the edges (**Figure 6**), but found no statistical differences for any toxin. For Accession 10917, the variance among the pileus samples was higher for phallacidin and beta-amanitin than for both alpha-amanitin and phalloidin. This is one of the first studies to examine variation within a single pileus. Our findings suggest that, if one were to select a random portion of the pileus, the sample would be fairly representative of alpha-amanitin and phalloidin levels, but less predictive for beta-amanitin and phallacidin.

We then analyzed dozens of mushrooms from multiple sites collected in 2015, and found surprisingly little difference in toxin levels between the different compartments (**Figure 7**). Generally, for all sites but the Pet site, the volva contained the most toxin, followed by the stipe, and then the pileus. These data strongly contrast with most previous work on European mushrooms of *A. phalloides*: Garcia et al 2015 found that in mushrooms collected in Portugal, amatoxins were concentrated in the pileus, while phallotoxins were highest in the volva (Garcia et al. 2015); Enjalbert found the same trend of mushrooms collected from several sites in France (Enjalbert et al. 1996) (**Table 2**). Perhaps, in California, *A. phalloides*-associated species that negatively impact its fitness are notably different from its natural enemies in Europe, and these enemies impact different compartments of the mushroom. The volva may protect more against soil-dwelling invertebrates and microbes, while the toxins of the pileus may be targeted at flying invertebrates such as flies in the *Diptera*. Future work could incubate the compartments separately to see which invertebrates, if any, emerge from the different compartments.

At the site “Pet,” the above trend was reversed: for each toxin, the most toxin was in the pileus, followed by stipe, and then volva (**Figure 7**). The toxin values at Pet were also much lower than at the Drake sites. Many potential factors could be driving the differences in toxin levels between these two areas. The Drake sites are 1200 m directly west of Pet, several hundred feet higher in elevation; Pet is situated on the Horsehead walking trail, nearer a creek, while the Drake sites are in more intact forest, and near a major road. Though Pet is a more recently established field site, without historical monitoring data, we cannot conclude whether *Amanita phalloides* recently arrived at Pet, or whether the species has been fruiting there for many years. Additional studies should elucidate site factors such as soil type, plant diversity, and soil microbial diversity, and whether such traits influence toxin distribution. Collection sites have been shown to correlate with toxin composition (Enjalbert et al. 1996), but the precise factors behind this influence remain unknown.

We found toxin levels varied from year to year (**Figure 8**), but contrary to our hypothesis, for three of the four toxins, levels increased from one year to the next.

In California, *A. phalloides* may be under selection to increase toxin production. This increase could be because native species that associate with *Amanita phalloides* are co-evolving tolerance to amatoxins, phallotoxins, or both. Some evidence suggests that, in mycophagous *Drosophila*, tolerance to amatoxins may be due to general toxin resistance such as cytochrome p450 production, and not mutations in RNA polymerase II (Stump et al. 2011). It would be informative to isolate insects associated with *Amanita phalloides* from sites in California to test whether they are resistant to amatoxins. Similarly, because the hyphae of *Amanita phalloides* is known to contain amatoxins (Kaya et al. 2013), although in lower amounts than is found in the mushroom, future studies could explore whether other ectomycorrhizal fungi in California sites containing *A. phalloides* possess tolerance to amatoxins or phallotoxins.

The increase in toxin values from one year to the next is particularly compelling in conjunction with the comparison of these California data with published European values. For alpha-amanitin in both the pileus and the stipe, the highest concentrations of California specimens are higher than any of the European values (**Figure 7, Table 2**). Perhaps the mushrooms in California originated in an area of Europe not covered by the studies listed here, such as the United Kingdom. Another possibility is that there has been selection for *A. phalloides* to increase toxin levels in California since its introduction. Additionally, differences in extraction protocol may play a role in this trend, and the protocol used here might be more efficient at removing toxin from tissues. However, the only other study known to us that used the same extraction protocol to compare alpha-amanitin levels in the introduced and native range of *A. phalloides* found a different pattern: a sample from the USA contained 0.88 mg/g alpha-amanitin, and the sample from Italy contained 1.33 mg/g (Sgambelluri et al. 2014). Our future work will utilize the same extraction protocol to make a direct comparison of toxin concentrations in the native range vs the introduced range.

Three primary factors could be driving this variation in toxin concentrations: weather, soil differences, and genetics. Our previous work suggests that toxins may readily leave mushroom tissue (Adams 2020; Bever et al. 2020). Rainfall may wash toxin from the mushrooms, such as the heavy rainfall that occurred during our 2015 sampling. However, our data indicate that for most toxins, rainfall actually increased from 2014, a drier year, to 2015, a relatively wetter year. Nonetheless, weather factors such as temperature and rainfall still may influence toxin expression. Secondly, Enjalbert et al 1996 found that soil type correlated with toxin production, and soil could be influencing toxin production here as well (Enjalbert et al. 1996). The Drake sites are classified as 135 (Inverness loam, on high slope) while Pet is 103 (Barnabe very gravelly loam) (Web Soil Survey). And yet, we found differences in toxin levels among the Drake sites, which share the same soil type. Furthermore, we saw variation in toxin from one year to the next (**Figure 8**), suggesting soil is less likely to be a primary driver of toxin variation.

We believe the variation in toxin concentrations is most likely due to genetic variation. Our recent work used Amplified Fragment Length Polymorphism (AFLP) and Whole Genome Sequencing to establish that, in both North America and Europe, genetically distinct individuals (genets) of *A. phalloides* are small and ephemeral, not seeming to last from one year to the next (Golan et al. 2019). Furthermore, this study showed that mushrooms located closer to each other are more closely related,

suggesting that spores settle and germinate close to their parent mushroom. Future work will examine whether genetic variation is sufficient to explain these differences in toxin concentrations between sites.

In conclusion, we found that toxin levels of *A. phalloides* in California are surprisingly variable, at each of the scales we examined. Some toxins like beta-amanitin and phallacidin are more variable within a mushroom, and the toxin concentrations across compartments are quite different from published values for European specimens. Future work should take inter-site variation into account when comparing across regions. Furthermore, toxin levels often vary from year to year, but not in the direction we hypothesized: generally, toxin levels increased from year to the next. We also found that, for alpha-amanitin, our highest toxin values in both cap and stipe were higher than any published values of European mushrooms. Taken together, these data suggest the amatoxins of *Amanita phalloides* may be under selection to increase toxin concentration. Instead of functioning as novel weapons, amatoxin and phallotoxin levels may be under rapid change, possibly increasing in order to protect the fungus from novel enemies.

3.4. Acknowledgements

The authors wish to thank Sydney Glassman and Lynn Boddy for their assistance with 2014 collections. Our sincere gratitude to the spectacular crew that helped map and collect samples in 2015: Jacob Golan, Holly Elmore, Susana Gonçalves, Natalia Vargas, Todd Mitchell, and more. And thank you to Sean Brown for many fun-filled trips to Point Reyes.

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