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Innate Chemical Resistance of Virginia Big-eared Bats (Corynorhinus townsendii virginianus) to White-Nose Syndrome

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Abstract:

White-nose Syndrome (WNS) is an emergent epidemic disease of bats in North America. Caused by the novel fungal pathogen *Pseudogymnoascus destructans*, with a mortality rate of >75%, in the last decade WNS has led to the local extinction of numerous bat species. Despite this high mortality, one species, the Virginia big-eared bat (*Corynorhinus townsendii virginianus*) remains unaffected. Virginia big-eared bats (VBEs) are commonly found covered in a yellow, oily substance, with a pelage commensal population dominated by the yeast, *Debaryomyces udenii*. As *D. udenii* is an oleaginous yeast that produces yellow colonies, the fungus may be responsible for the production of this oily substance on bats. In order to test this, 54 swab samples from the pelage of various bat species, including VBEs, were collected, along with cultures of *D. udenii* and the control yeast *Saccharomyces cerevisiae.* These samples were extracted using the Bligh and Dyer lipid extraction method and reversed-phase lipid chromatography to identify shared lipid metabolites. The data demonstrated that only a handful of lipids were unique to *D. udenii* (compared to *S. cerevisae*), and only seven of these lipid candidates were found on VBE pelage extracts. Instead of indicating that *D. udenii* was responsible for the production of the yellow material, our data suggests that the yellow material on bats is selecting for the presence of this yeast, possibly over filamentous fungi. As VBEs have large pararhinal glands, our hypothesis is that the material produced by these glands might be anti-fungal, selecting against the growth of filamentous fungi on the skin.

Introduction:

White-nose Syndrome (WNS) is a prevalent fungal disease of bats that has killed in excess of 5.7 million bats since its initial identification in 2006 [1]. WNS is caused by the novel fungal pathogen, *Pseudogymnoascus destructans*. This pathogen has been shown to cause mortality rates that can exceed 75% in infected bat populations [1]. Infection by this psychrophilic fungus normally takes place during hibernation when bats suppress their immune system, metabolism, and body temperature [2]. Hibernation provides the bats a way to conserve energy and survive the winter, but it provides the perfect opportunity for *P.destructans* growth at its optimal temperature range (1°C to 15°C) [3]. The fungus infects the wings of the bat, with pathogenesis of WNS occurring through an epidermal infection of the wings. Bats maintain water homeostasis during hibernation by water adsorption through the wings, so this wing damage leads to dehydration and electrolyte imbalance [4]. The loss of water causes the bat to become dehydrated, leading to arousal [5]. Arousal from hibernation forces the bat to expend crucial energy in search of a source of water, using fat reserves equivalent to 63 days of

hibernation for each arousal [6]. Without a source for water and with the continual loss of water, the bat continues to wake up during hibernation, eventually dying either of starvation or exposure on the landscape [6]. The spread of WNS is facilitated by the tendency of bats to cluster during hibernation, which provides the bats a way to expend less energy on thermal regulation, and uses the body heat of surrounding bats during hibernation [7]. Such clustering promotes the bat-to-bat transfer of *P. destructans.,* leading to the high infection and mortality rates seen in infected bat populations.

Within the United States the nine bat species susceptible to WNS are cave and mine hibernating species. These include the big brown bat (*Eptesicus fuscus*), the eastern small-footed bat (*Myotis leibii*), the gray bat (*Myotis grisescens*), the Indiana bat (*Myotis sodalis*), the little brown bat (*Myotis lucifugus*), the tri-colored bat (*Perimyotis subflavus*), the northern long-eared bat (*Myotis septentrionalis*), the southeastern bat (*Myotis austroriparius)*, and the Yuma bat (*Myotis yumanensis)* [8,9]. There are some species of mine and cave hibernating bat that are not susceptible to WNS, including the Virginia big-eared bat (*Corynorhinus townsendii virginianus*). This bat species is infected by *P. destructans* during hibernation, but does not demonstrate any pathology, despite other bat species residing in the same hibernacula dying in high numbers [10]. One unusual feature of Virginia big-eared bats (VBE's) is that they are commonly found covered in a yellow, oily substance, often referred to as gunk by bat biologists, that highly resembles a waxy substance (Figure 1). This yellow material is unique to two bats within the *Corynorhinus* genus, including the Virginia big-eared bat and the Rafinesque's big-eared bat, both of which do not succumb to WNS.

Figure 1: A swab showing the yellow, waxy material taken from the pelage of a Virginia big-eared bat.

Commensal microorganisms are diverse in the benefits that they provide to their hosts. Within the bodies of mammals, beneficial commensals play an essential role in the gastrointestinal tract, aiding in the digestion of food [11]. On the epidermis of mammals, commensals contribute to the regulation of skin chemistry. They are also an important part of the innate immune system, outcompeting other microorganisms for necessary resources, signaling the host's adaptive immune system to the presence of a possible foreign microorganisms, and producing antimicrobials capable of destroying harmful microorganisms [12,13]. We therefore wondered whether the yellow material was being produced by a commensal species on the pelage of the bat.

To determine the commensal populations that inhabited the pelage of the VBEs, a graduate student, Kelsey Njus, examined the fungal population of different bat species (Figures 2-3) [9]. The most prevalent fungi identified on VBE bats was the yeast, *Debaryomyces udenii,* respectively 54% of all OTUs identified and as much as 94% of the community for one bat [9].

The most common fungi found on the VBEs were cultured and it was noted that *D.udenii* produced a distinct yellow colonies. *D. udenii* is an oil-producing, oleaginous species, and the yellow provoked the idea that *D. udenii* could be the commensal responsible. My honors research project was to determine whether this species was responsible for the production of the yellow gunk.

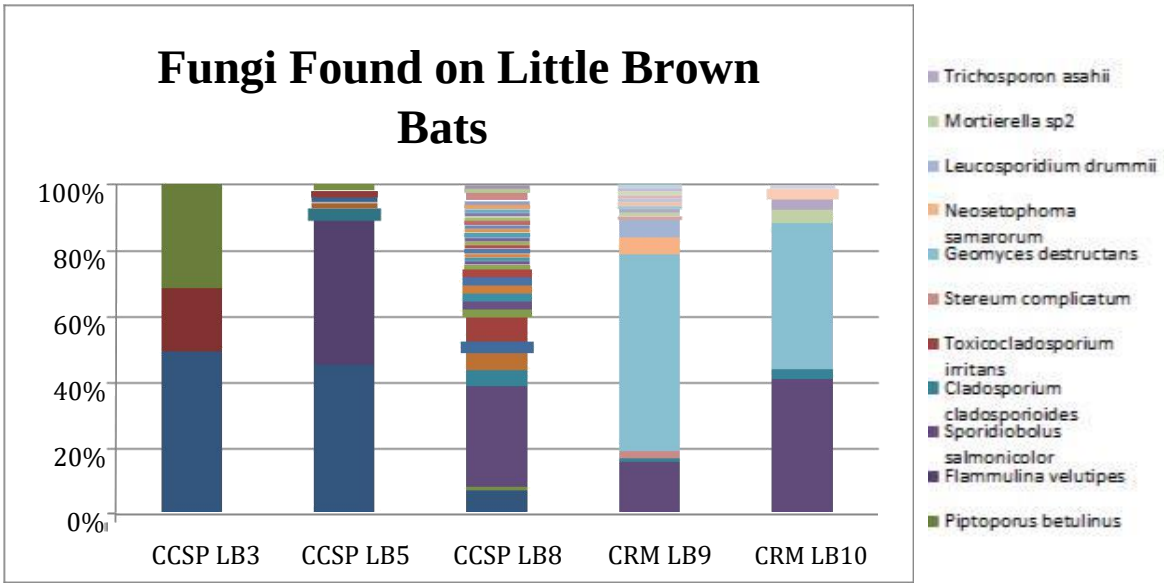

Figure 2: A comparison of the fungal OTUs identified on all little brown bats (LBs) sampled. CCSP = Canoe Creek State Park; CRM = Casselman River Mine. The legend to the right of the figure denotes the species which make up large proportions on individual or shared fungal community profiles [9]. Courtesy of K. Njus.

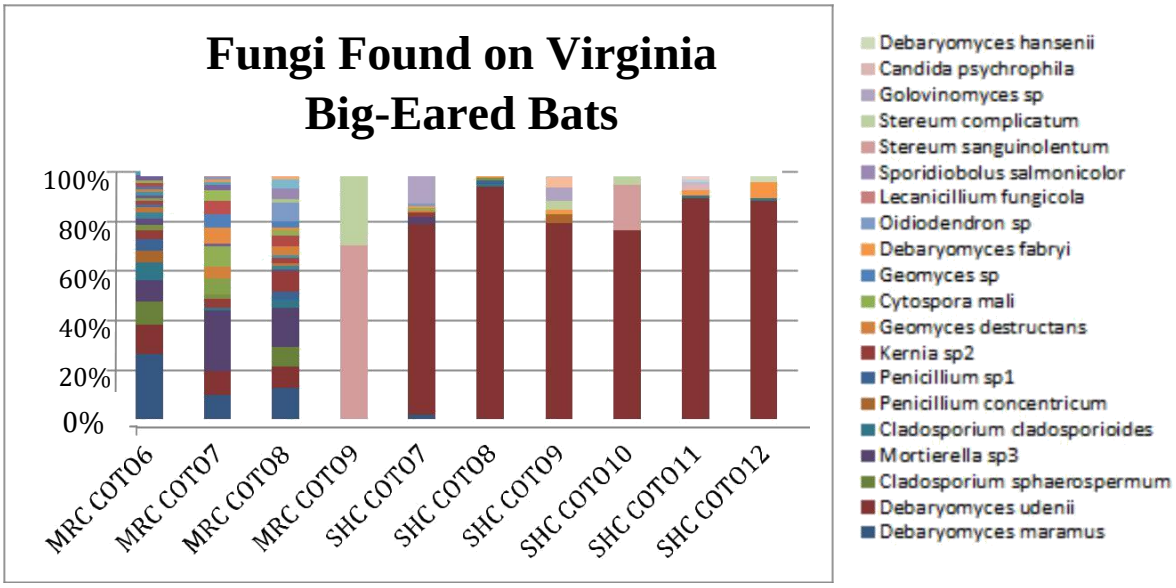

Figure 3: A comparison of the fungal OTUs identified on all Virginia big-eared bats sampled. MRC = Minor Rexrode Cave; SHC = Schoolhouse Cave. The legend to the right of the figure denotes the species which make up large proportions on individual or shared fungal community profiles [9]. Courtesy of K. Njus.

Materials and Methods:

Sample Collection

Collection was carried out using non-invasive techniques using a sterile cotton swab (Figure 4). Sample collection was performed by Mr. Craig Stihler and his colleagues at the Virginia Department of Natural Resources during the normal seasonal count to avoid unnecessary disturbance of the endangered VBEs. To avoid sample degradation, swabs were stored at -20°C after collection.

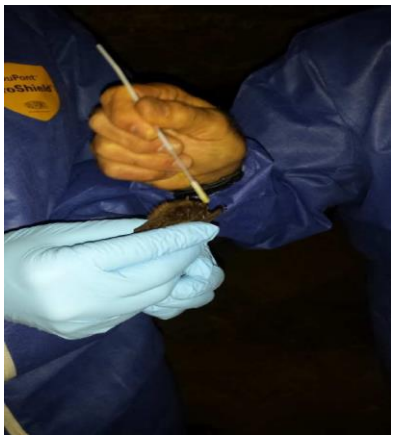

Figure 4: The yellow material was collected non-invasively using a cotton swab.

Growth Conditions

Debaryomyces udenii ATCC 66545 and *Saccharomyces cerevisiae* ATCC 9763 were each obtained from the American Type Culture Collection and cultured in 125mL of Potato Dextrose Broth at 30°C. Yeast growth was confirmed by Gram stain. 1 mL of cultured yeast was added to a 1.5 mL Eppendorf tube and then centrifuged for 20 minutes at 3,500 rpm to harvest cells. After centrifugation, supernatant was removed and 1 mL of culture was added to the newly formed pellet. This process was repeated until each pellet of cells was approximately 0.2 mL in volume.

Lipid Extraction and Chemical Analysis

Swab samples of the pelage of the little brown bat (*M. lucifugus*) were used as a negative control to contrast the swab samples from the Virginia big-eared bats. *S. cerevisiae,* a common brewing yeast, was used as a negative control.

Six swab samples from the pelage of VBE bats and 5 swab samples from the little brown bats were subjected to a modified version of Bligh and Dyer lipid extraction method [14]. Each swab tip was individually submerged in a 1:2 (v/v) solution of HPLC grade chloroform (Alfa Aesar, Ward Hill, MA) and HPLC grade methanol (Merck Millipore, Burlington, MA) for 30 – one second intervals. 0.250 mL of HPLC grade chloroform was then added to each sample, then vortexed for 30 seconds. 0.250 mL of HPLC grade water (G.E. Life Sciences, Logan, Utah) was added to each sample and then vortexed on high for 30 seconds. Samples were then frozen at -20°C for one hour and then centrifuged at 5,000 rpm in a table top centrifuge for 5 minutes at 4°C to create a two-phase system. The organic layer was then recovered and then dried to concentrate using a Savant SVC-100H speedvac concentrator (Thermo Fisher Scientific, Columbus, Ohio).

D. udenii and *S. cerevisiae* samples (ten 0.2 mL pellets of each) were subjected to the same protocol, but were first subjected to cellular lysis. To do this, yeast pellet samples were placed in 0.1 mL of HPLC grade methanol. The pellet submerged in methanol was first vortexed for 30 seconds, then frozen in liquid nitrogen for 30 seconds, thawed, and finally sonicated for 2 minutes. This was then repeated five times for each sample. 0.75 mL of a 1:2 (v/v) solution of HPLC grade chloroform (Alfa Aesar, Ward Hill, MA) and HPLC grade methanol (Merck Millipore, Burlington, MA) was then added to the lysed cells. Followed by 0.250 mL of HPLC grade chloroform. The sample was then vortexed for 30 seconds. 0.250 mL of HPLC grade water (G.E. Life Sciences, Logan, Utah) was added to each sample and vortexed on high for 30 seconds. Samples were placed in -20°C for one hour and then centrifuged at 5,000 rpm in a table top centrifuge for 5 minutes at 4°C to create a two-phase system. The organic layer was then recovered and dried on the speedvac concentrator.

Reversed-phase liquid chromatography was performed on the concentrated organic phases from all four sample types using a Kinetex 5µm EVO C18 column followed by analysis on a SCIEX Triple-Time of Flight mass spectrometer with an Eksigent MicroLC 200 system (AB SCIEX, Concord, Ontario, Canada).

Results:

To determine if there were unique lipids on VBE bats, I extracted lipids from pelage swab samples from 54 separate bats (Table 1). These bats included VBE bats, Rafinesque's big-eared bats (CORA), Northern long-eared bats (MYSE), little brown bats (MYLU), big brown bats (EPFU), and tri-colored bats (PESU) as controls. All of these species, are infected by *P. destructans;* however, VBE and CORA bats do not demonstrate any WNS pathology. The samples were collected from numerous caves in Kentucky, West Virginia, and Pennsylvania to ensure than any differences were not geographic. The samples extracted are described in Table

1. I also extracted lipids from *D. udenii* and *S. cerevisae*, using *S. cerevisae* as a negative control.

The reversed-phase liquid chromatography yielded complete lipid profiles for the sample types, yielding thousands of MS peaks. Lipid candidates were cross-referenced to identify any shared candidates; in addition to first removing any lipid candidates that were shared with the negative controls. The lipid profile of *D. udenii* was first cross-referenced against the lipid profile of *S. cerevisiae* to determine which lipids were unique to *D. udenii*. Then, the lipid profile of the swab data from the VBE pelage was compared to swab data of the little brown bat, the negative control, to determine any common lipids that could be eliminated, leaving 7,755 candidate metabolites. Finally, the lipid candidates unique to the *D. udenii* were compared with the unique lipid profiles from the VBE bat, identifying seven unique lipid candidates (Figure 5). The retention profiles of these seven candidate lipids is shown in Table 2; however, this data from the reversed-phase liquid chromatography did not yield any candidate identities. In order to increase our ability to identify these candidates, fragmentation using the Triple-TOF mass spectrometer was performed (Figures 6 and 7); however, the fragmentation data did not provide additional information on the structure of these molecules.

Species	Location	Date Collected	Type of Sample	Sex of Bat	Weight (mg)	Date Extracted
VBE	School House	2/18/2015	Swab	NA	NA	1/12/2016
VBE	School House	2/18/2015	Swab	NA	NA	1/12/2016
VBE	School House	2/18/2015	Swab	NA	NA	1/12/2016
VBE	School House	2/18/2015	Swab	NA	NA	1/12/2016
VBE	School House	2/18/2015	Swab	NA	NA	1/12/2016
VBE	School House	2/18/2015	Swab	NA	NA	1/12/2016
CORA	Kentucky	9/13/2009	Swab	NA	NA	8/28/2016
CORA	Kentucky	9/13/2009	Swab	NA	NA	8/28/2016
CORA	Kentucky	8/26/2009	Swab	NA	NA	8/28/2016
CORA	Kentucky	9/13/2009	Swab	NA	NA	8/28/2016
CORA	Kentucky	9/13/2009	Swab	NA	NA	8/28/2016
CORA	Kentucky	9/13/2009	Swab	NA	NA	8/28/2016
EPFU	Kentucky	8/25/2009	Swab	NA	NA	11/15/2017
EPFU	Kentucky	8/25/2009	Swab	NA	NA	11/15/2017
EPFU	Kentucky	9/13/2009	Swab	NA	NA	11/15/2017
EPFU	Kentucky	8/26/2009	Swab	NA	NA	11/15/2017
MYLU	Kentucky	9/13/2009	Swab	NA	NA	11/27/2017
MYSE	Kentucky	8/26/2009	Swab	NA	NA	11/27/2017
VBE	Bowman Salt Peter - Jackson County	2/3/2011	Swab	Male	NA	9/25/2017
MYLU	Dunmore Mine, PA	10/30/2009	Swab	NA	NA	8/28/2016
MYLU	Dunmore Mine, PA	10/30/2009	Swab	NA	NA	8/28/2016
MYLU	Dunmore Mine, PA	10/30/2009	Swab	NA	NA	8/28/2016
MYLU	Dunmore Mine, PA	10/30/2009	Swab	NA	NA	8/28/2016
MYLU	Dunmore Mine, PA	10/30/2009	Swab	NA	NA	8/28/2016
MYLU	Carter Cave	NA	Hair	NA	2.3	10/30/2017
MYLU	Carter Cave	NA	Hair	Male	0.8	10/30/2017
MYLU	Carter Cave	NA	Hair	Male	2.9	10/30/2017
PESU	Carter Cave	NA	Hair	Male	0.9	10/30/2017
MYSE	Carter Cave	NA	Hair	Female	0.8	10/30/2017
CORA	Currie Cave	1/13/2011	Hair	Female	3	10/2/2017
CORA	Currie Cave	1/13/2011	Hair	Female	6.3	10/2/2017

Species	Location	Date Collected	Type of Sample	Sex of Bat	Weight (mg)	Date Extracted
CORA	Currie Cave	1/13/2011	Hair	Female	4.4	10/2/2017
CORA	Currie Cave	1/13/2011	Hair	Female	7.6	10/2/2017
CORA	Currie Cave	1/13/2011	Hair	Male	8.8	10/2/2017
CORA	Currie Cave	1/13/2011	Hair	Male	9.3	10/2/2017
CORA	Currie Cave	1/13/2011	Hair	Male	7.3	10/2/2017
VBE	Sinnett Cave	9/3/2014	Pararhinal Gland	NA	~0.004	3/7/2017
VBE	Sinnett Cave	9/3/2014	Pararhinal Gland	NA	~0.002	10/10/2016
CORA	Hickory Flats (Room 3)	1/26/2018	Swab	NA	NA	4/2/2018
CORA	Hickory Flats (Room 3)	1/26/2018	Swab	NA	NA	4/2/2018
CORA	Hickory Flats (Room 1)	1/26/2018	Swab	NA	NA	3/30/2018
CORA	Hickory Flats (Room 1)	1/26/2018	Swab	NA	NA	3/30/2018
CORA	Currie Cave	2/6/2018	Swab	NA	NA	3/30/2018
CORA	Currie Cave	2/7/2018	Swab	NA	NA	3/30/2018
CORA	Wildcat Hollow	2/14/2018	Swab	NA	NA	3/30/2018
CORA	Wildcat Hollow	2/14/2018	Swab	NA	NA	3/30/2018
CORA	Wildcat Hollow	2/14/2018	Swab	NA	NA	4/2/2018
CORA	Wildcat Hollow	2/14/2018	Swab	NA	NA	4/2/2018
CORA	Wildcat Hollow	2/14/2018	Swab	NA	NA	3/30/2018
CORA	Wildcat Hollow	2/14/2018	Swab	NA	NA	3/30/2018
CORA	Peter Branch	2/14/2018	Swab	NA	NA	3/30/2018
CORA	Peter Branch	2/14/2018	Swab	NA	NA	3/30/2018
CORA	Peter Branch	2/14/2018	Swab	NA	NA	3/30/2018
CORA	Peter Branch	2/14/2018	Swab	NA	NA	3/30/2018

Table 1: The types and locations of the bat samples that have been subjected to Bligh and Dyer Lipid Extraction and reversed-phase liquid chromatography throughout the entirety of my honors research project. Rafinesque's big-eared bat (CORA), Virginia big-eared bat (VBE), Northern long-eared bat (MYSE), Little brown bat (MYLU), Tri-colored bat (PESU), Big brown bat (EPFU).

m/z	retention time (min)
313.22	30.95
327.07	51.70
343.27	46.90
391.27	49.81
391.33	0.29
409.20	0.05
517.38	1.19

Table 2: LC-MS/MS data of the candidate lipid molecules.

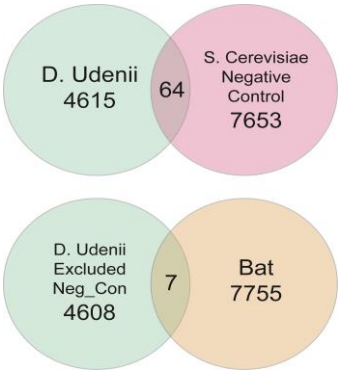

Figure 5*:* The cross-reference of each type of sample, ultimately leading to only seven unique candidates being shared between the *D. udenii* and the sample of the yellow material from the VBE pelage.

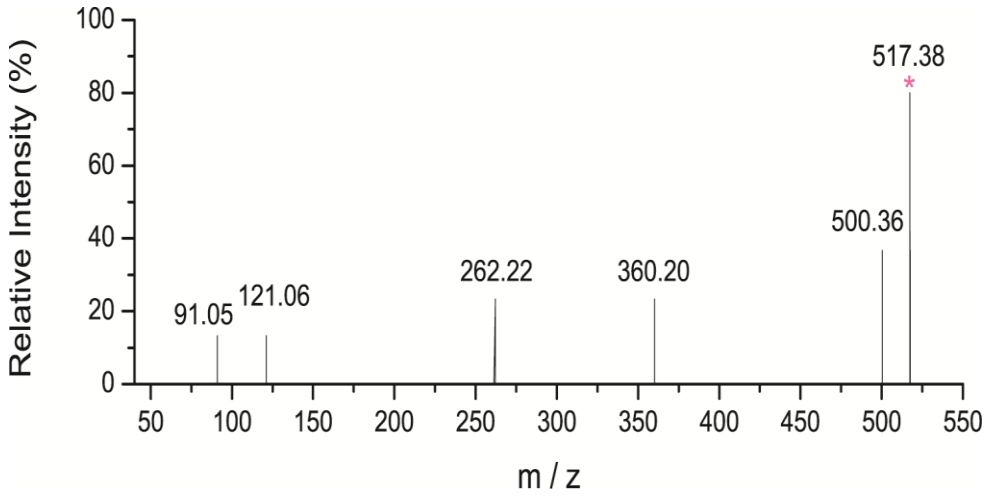

Figure 6: Fragmentation data (MS/MS) generated for candidate molecule m/z = 517.38.

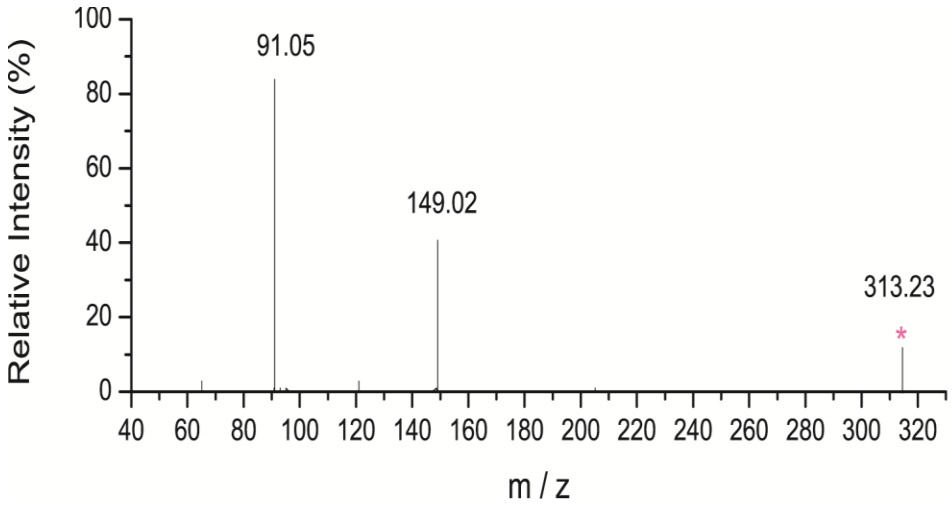

Figure 7: Fragmentation data (MS/MS) generated for candidate molecule m/z = 517.38

To aid in the identification of these samples, reversed-phase liquid chromatography was performed on an additional 25 samples of extracted *D. udenii* lipids. This large scale data set yielded a stronger baseline for the lipids shared between *D. udenii* and the yellow gunk, but the spectra of the seven candidates were shown to have diminished strength from the first round of testing. This lack of signal strength from the seven spectra, according to the new baseline, provided a lowered confidence in their significance as the lipids that were producing the antifungal activity – in theory the *D. udenii* is producing so much of this material on the bat that the bat feels oily when you pick it up, suggesting that the signals we identified for these compounds should have been very strong.

As a result, I decided to shift my focus away from the commensal yeast, and look at the role of the pararhinal gland found within both the VBE and Rafineque's big-eared bats. This gland, located in the nose, secretes a fluid that these two species of bats coat themselves in during the grooming process. Thus, the pararhinal secretion may be inhibiting the growth of filamentous fungi, such as *P. destructans*, and allowing yeast fungi to become the dominant fungi on these species. I therefore used the modified Bligh and Dyer Lipid Extraction to extract lipids from a VBE pararhinal gland and compared this to the lipid profile of the yellow gunk. The two sample types had multiple candidates in common and the three strongest spectra were chosen as possible candidates (Figures 8-10). Of these, the lipid with a retention rate of m/z 315.26 (Figure 8) was thought to be the most likely candidate due to its epoxide functional group. The reactivity provided by such a functional group may play a role in the mechanism underlying an antifungal agent.

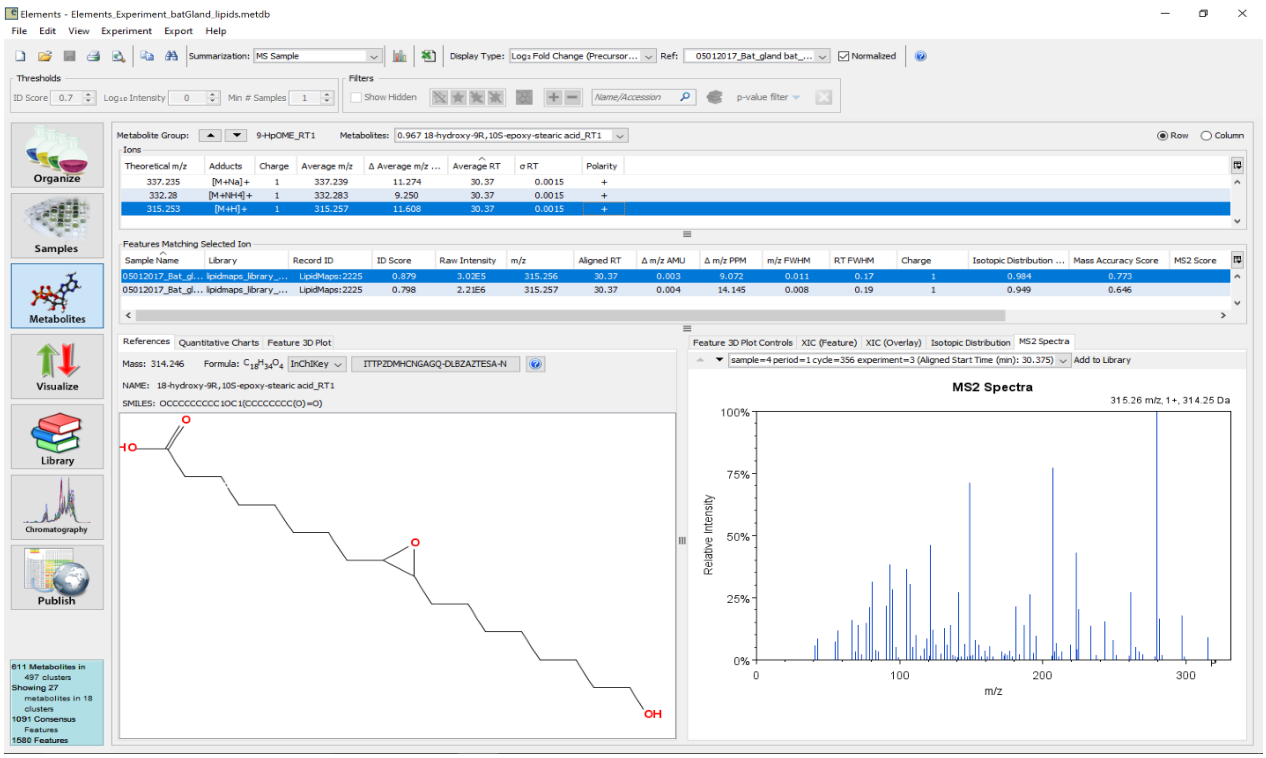

Figure 8: The MS/MS spectra and possible structure of candidate m/z = 315.26 from the VBE pararhinal gland.

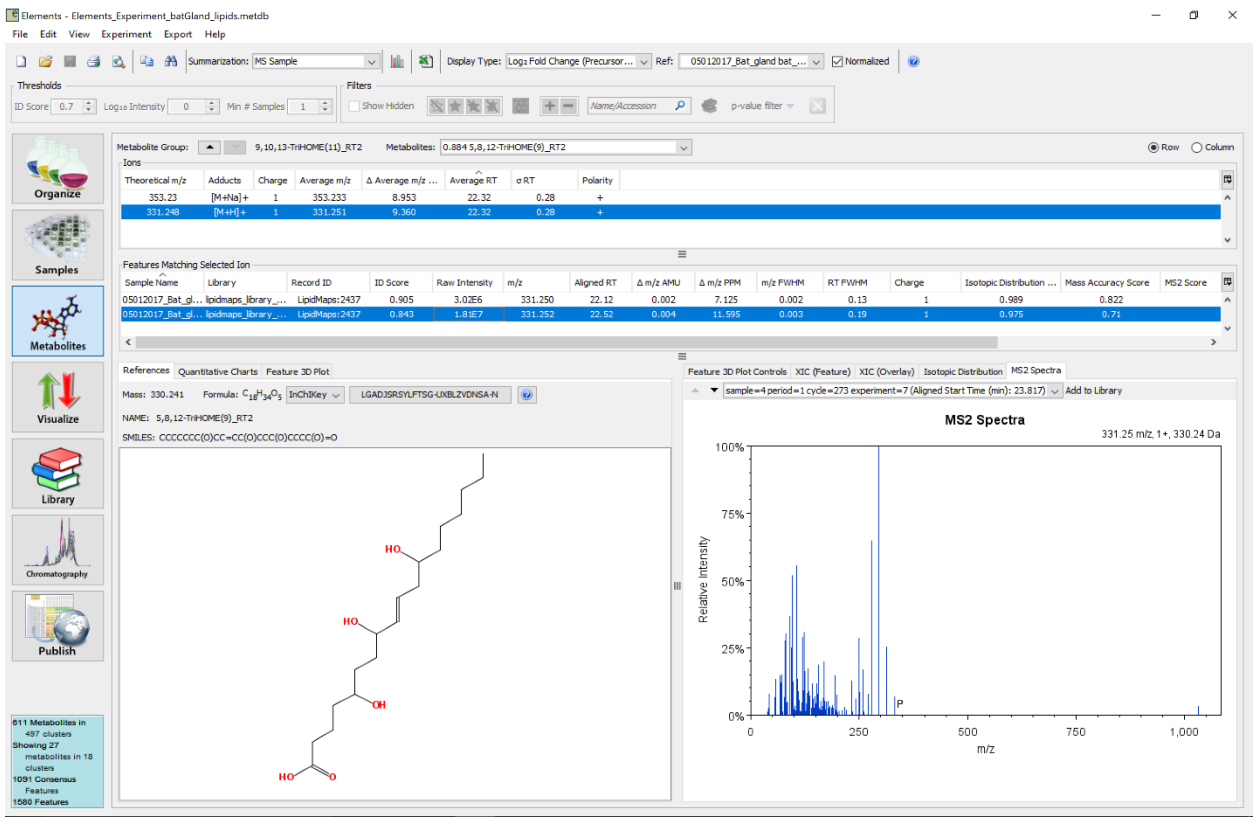

Figure 9: The MS/MS spectra and possible structure of candidate m/z = 331.25 from the VBE pararhinal gland.

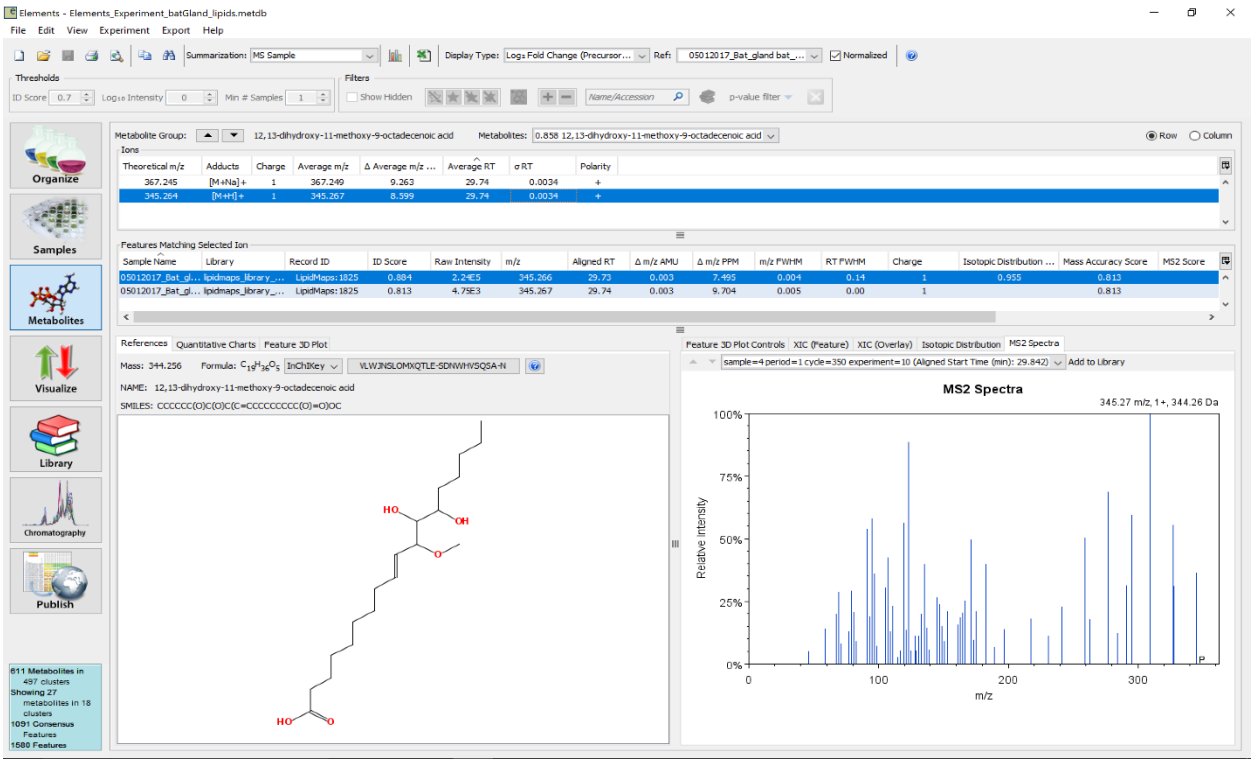

Figure 10: The MS/MS spectra and possible structure of candidate m/z = 345.27 from the VBE pararhinal gland.

Discussion

With so many bats succumbing to WNS, it is unclear as to why the VBE and CORA bats, infected by *P. destructans*, have not been developing WNS pathology, even as they are hibernating in caves with significant bat die-offs. One of the characteristics that bat researchers have identified, which appears to be unique to both species, is the presence of a yellow, waxy substance that makes the bats feel oily or greasy when handled. We wondered whether this yellow material could be responsible for the unique resistance found in these species to WNS.

Previous work by K. Njus in the Barton Lab had identified a fungus that dominated the pelage of all VBE tested, which came from a genus known to over-secrete lipids. When grown on media, this yeast species produced a bright yellow colony, which made me think that this species *D. udenii* was producing the yellow gunk. The common yellow color of the oil-producing yeast and the waxy nature of the gunk led us to believe that the antifungal agent may be a lipid compound. After the reversed-phase liquid chromatography, seven candidate lipids were found to be shared by *D. udenii* and the yellow gunk, yet absent in both negative controls. Unfortunately, the identification of these seven compounds was un-reproducible, suggesting that maybe the unique antifungal compound might not come from *D. udenii*, especially as the fungus would need to be producing so much of it that bats would feel oily/waxy when held.

We are therefore looking at expanding the scope of the research to compare the lipids from VBE and CORA bats and examine the pararhinal glands of these bats. Lipid extractions revealed much more dominant lipids in these glands that are common with the yellow gunk found on bats. MALDI FT-ICR MS are being performed on the pararhinal gland of these bats to determine if these compounds are found within the glands. These new samples will shed light on the possible similarities between the lipids of these two species of bats, and whether they produce antifungal lipids. We believe that these lipids may be preventing the growth of filamentous fungi, which would explain the dominance of yeast on their pelage.

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