Advancing Analytical Techniques to Study Biology: Mass Spectrometry Based Analysis of Small Molecules

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ABSTRACT

Mass Spectrometry has become a powerful tool to study biological systems. Tailoring detection strategies to specific classes of molecules improves discovery-based investigations. This thesis will investigate three classes of compounds in three distinct biological groups. The first study that will be discussed was examining the phospholipidome of *Candida Albicans* to investigate virulence. The second study was monitoring isotopically labeled metabolite flux of coral infected with black band disease to study how disease progression affects metabolic rates. Finally, a novel method was developed to examine the composition of toxic steroids that fireflies use for protection.

These discovery-based studies demonstrate the capabilities of mass spectrometric studies to probe biologically relevant molecules. Discovery based studies can generate new drug targets, corroborate previous findings, and detect unknown molecules which drive metabolism changes.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The small molecule composition provides information about cells or systems on the molecular level. ‘Omics techniques aim to identify the total composition of target small molecules in a sample. The “metabolome” of a cell refers to the total amount of small molecules. “Metabolomics” refers to the study of the composition of small molecules. Metabolomics aims to study this small molecule space by measuring the totality of small molecules in a cell or system.[1] These small molecules include water soluble metabolites, non-polar phospholipids, and steroids.

Metabolomics tells us about the final state of a cell after gene expression, post-translational modification, and its environmental conditions. Studying the cell at the gene level or the protein level tells us what the cell wants to do; however, if we look at the composition of small molecules in the cell, it tells us what the cell is actually doing.[2] Other ‘omics techniques such as genomics, proteomics, and transcriptomics look at what a system can do or what a system is trying to do. Metabolomics, on the other hand, takes into consideration post-translation modifications that can happen in response to genetic mutations or environmental stressors.[3] By studying the small molecule space—which consists of compounds below 1,000 Daltons—we can see the actual state of the system. Within this small molecule space, there is vast chemical diversity.

Mass-spectrometry–based analysis have become a valuable tool in systems biology. Mass spectrometers measure the mass to charge (*m/z*) ratios of ionized compounds, and this allows us to study the small molecule space. All of the studies discussed herein were completed using a Thermo Scientific Exactive Plus Orbitrap. Orbitrap mass analyzers measure the path of ions around a charged spindle leading to high mass accuracy and low limits of detection.[4] Exactive Plus Orbitraps also have an HCD cell which can apply voltage to induce fragmentation. This is termed “all-ion fragmentation” because all of the ions are fragmented, as opposed to a triple quadrupole mass spectrometer, which fragments based on selected parent masses. The ionization

sources most commonly used for small molecules studies are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI).[5] Coupling liquid chromatography with mass spectrometry improves detection by separating ions by their chemical properties before the ions are introduced to the mass spectrometer. This decreases the load on the mass analyzer to improve detection. Liquid chromatography–mass spectrometry (LC-MS) methods are also beneficial because when compounds are separated prior to analysis they yield information in the mass domain as well as the time domain.[6]

Mass spectrometers have greatly improved our ability to study the chemical makeup of substances and organisms. The *m/z* ratio of a compound depends on the chemical structure of the molecule. Mass spectrometry (MS) methods vary depending on what molecules they are aimed at detecting. “Untargeted” MS methods aim to measure all known and unknown ions in a sample. Positive identifications are made using exact mass and retention times for high-performance liquid chromatography (HPLC) methods.[7] The benefit to this approach is that it enables the discovery of new molecules; however, the large chemical diversity presents challenges. Of course, certain classes of compounds have properties that make them more detectable than others, which can obscure less-detectable molecules in a largely diverse chemical environment. In contrast to untargeted MS methods, targeted MS methods look at a specific subset of chemicals with similar properties. Chemical standards are used for absolute quantitation. The benefit of this method is absolute quantitation, but the drawback is the inability to discover new molecules. Somewhere in between these two approaches are semi-targeted MS methods.[8] Sample preparation, chromatography, and processing are all tailored to a set of compounds with similar properties. Results yield relative quantitation so that the abundance of particular ions can be compared across samples. The benefit to semi-targeted approaches is that the specific molecules of interest can be positively identified with relative quantitation without relinquishing the possibility of new molecules involved in cellular processing being discovered.[9] The three methods discussed in this thesis- lipidomics, fluxomics, and an HPLC/PDA/MS method for lucibufagin detection- can be considered semi-targeted methods.

“Lipidomics” focuses on the non-polar lipids in a sample. Lipids make up the cellular membrane, hold proteins in place, and act as signaling or transport molecules. Phospholipids in particular are of great interest to biologists studying various diseases and environmental condition adaption.[10] Cellular membranes consist of a phospholipid bilayer with hydrophobic fatty acid tails on the inside of the membrane and the outside is made of up the hydrophilic phospholipid head groups. The composition of the phospholipid membrane depends on the type of cell, its state, and its surrounding conditions.[11] The tails are carbon chains with potentially varying degrees of unsaturation. The degree of saturation affects the rigidity of the membrane.[12] The head groups can group together and rearrange the phospholipid compositions based on the cell’s needs.[13] The phospholipid membrane has many functions which makes it a desirable target for studying a variety of organisms and disease states. Lipidomics analysis was used to elucidate the impact of phosphatidylserine synthase on virulence in *Candida albicans*.

“Fluxomics” is a subfield of metabolomics and is the study of the rate of turnover of metabolites.[14] By growing cells using an isotopically labeled substrate we can measure how long it takes for the substrate to travel through established metabolic pathways. Kinetic rates can then be calculated based on isotope incorporation.[15] As discussed, metabolomics looks at the composition of small molecules of a cell. Metabolomics yields helpful information yet does not reveal the rate at which the metabolome is changing. The rate at which metabolites are progressing through metabolic pathways can yield important insights into phenotypic differences, environmental adaptions, and virulence. However, the isotope labeling utilized in fluxomics allows us to observe how the rate of turnover for metabolites changes in response to their environment or disease.[16] An isotope labeling experiment on coral infected with black band disease was completed to investigate virulence effects on metabolite turnover rates.

A semi-targeted method for lucibufagin detection was developed using high-performance liquid chromatography, a photodiode array detector (PDA), and an Orbitrap MS. Lucibufagins (LBGs) are a group of cardiotonic steroids that fireflies use

as a chemical defense against predators.[17] All LBGs share a specific steroid backbone structure but differ in their substituent groups and positions. An HPLC/PDA/MS method was developed to detect known LBGs as well as to potentially identify unknown LBGs. A PDA was used to identify unknowns because LBGs are UV active compounds that absorb at 300nm. Almost 700 firefly samples were run on the HPLC/MS method and then a subset of those showing the most LBG diversity was run in-line with a photodiode array detector to obtain a list of possible unknown LBGs.

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CHAPTER 1
ROLE OF PHOSPHATIDYLSERINE SYNTHASE IN SHAPING THE PHOSPHOLIPIDOME OF *CANDIDA ALBICANS*

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ATF finalized the lipidomics mass spectrometry method, extracted and ran the samples, analyzed the mass spectrometry data, and contributed to writing the paper. CDC designed the experiment, grew the cultures, did the assays and enzyme kinetics and wrote the paper.

Abstract

Phosphatidylserine (PS) synthase (Cho1p) and the PS decarboxylase enzymes (Psd1p and Psd2p), which synthesize PS and phosphatidylethanolamine (PE), respectively, are crucial for *Candida albicans* virulence. Mutations that disrupt these enzymes compromise virulence. These enzymes are part of the cytidine diphosphate-diacylglycerol pathway (i.e. de novo pathway) for phospholipid synthesis. Understanding how losses of PS and/or PE synthesis pathways affect the phospholipidome of *Candida* is important for fully understanding how these enzymes impact virulence. The cho1Δ/Δ and psd1Δ/Δ psd2Δ/Δ mutations cause similar changes in levels of phosphatidic acid, phosphatidylglycerol, phosphatidylinositol and PS. However, only slight changes were seen in PE and phosphatidylcholine (PC). This finding suggests that the alternative mechanism for making PE and PC, the Kennedy pathway, can compensate for loss of the de novo synthesis pathway. *Candida albicans* Cho1p, the lipid biosynthetic enzyme with the most potential as a drug target, has been biochemically characterized, and analysis of its substrate specificity and kinetics reveal that these are similar to those previously published for Saccharomyces cerevisiae Cho1p.

Introduction

Lipid Function

Phospholipids consist of a hydrophilic head group and hydrophobic tails. They are arranged in two parallel layers with the hydrophobic tails facing each other and the head groups facing out. This creates the phospholipid bilayer that makes up cellular membranes and hold proteins in place. The phospholipid composition affects the physical properties of the cell membrane (Milne et al. 2006). The importance of the cellular membrane to healthy functioning of cells has made phospholipid synthesis pathways popular targets for drug therapies. The phospholipid head groups can be phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG), and phosphatidic acid (PA) (Sohlenkamp and Geiger 2016). Cells use phospholipid synthesis pathways to rearrange the composition of phospholipids to meet their needs. The fatty acid tails typically contain between 12 to 22 carbons with no or multiple degrees of unsaturation or double bonds (Shevchenko and Simons 2010). The length and number of double bonds in the fatty acid tails affect the membrane as well and cells can reorder and rearrange these as needed.

Membrane lipids have an asymmetric distribution and are in a dynamic state since cells can change their membrane composition to adjust to their environment . The specific distribution of the phospholipids in the membrane is important to cellular function (van Meer et al. 2008). Therefore, phospholipid synthesis pathways are effective targets for drug therapies aimed to decrease the virulence of pathogens.

Lipidomics Method Development

Electrospray Ionization

For analytes to enter the mass spectrometer for analysis they must be charged and in the gas phase. There are many ionization techniques that can be coupled to mass spectrometry but the preferred ionization source for detecting lipids is electrospray

ionization (ESI) (Peterson and Cummings 2005). ESI is a soft ionization technique that doesn't induce fragmentation and yields mostly singly or doubly charged ions for lipids. The lack of ion source fragmentation and the ions ESI produces has made ESI the preferred ionization source used for lipidomics. Lipidomics studies that don't incorporate separation via liquid chromatography prior to analysis are referred to as "shotgun" lipidomics. Liquid chromatography separation techniques are beneficial to lipid studies to decrease ion load on the detector as well as provide retention time information for the analytes (Li et al. 2011). For LC-ESI-MS experiments, the eluent from the column is sprayed through a charged nozzle. The spray is dried by N_2 gas and the nozzle can be heated to assist drying. As these charged liquid droplets dry, they shrink and the ions get closer and closer together until their electrostatic repulsions finally repel them into gaseous ions (Fenn et al. 1990). These ions then enter the mass spectrometer via an ion transfer tube and enter the low pressure first stage of the instrument. The ESI probe can be operated in positive or negative mode to match the detection mode of the mass spectrometer. The composition of the mobile phase chosen for the chromatographic separation can help or hinder ionization depending on the chemical composition of the analytes of interest (King et al. 2000).

Chromatography

Liquid chromatography (LC) is an analytical technique used to separate compounds based on their chemical properties. The stationary phase is packed into a column and liquid mobile phases carry the analyte through the column. Molecules are retained on the column due to molecular interactions between the analyte in the mobile phase and the stationary phase (Coskun 2016). The retention time of a compound can be used for validation since the chromatography gradient can be optimized to separate certain classes of molecules. LC-MS studies collect information on molecules in the mass domain as well as the time domain. Another benefit of pairing LC to MS is that fewer molecules will be entering the mass spectrometer at a given time which decreases the load on the detector which can increase sensitivity to improve detection.

The most common types of LC used for small molecule analysis are normal phase chromatography, reverse phase chromatography, and hydrophilic interaction

chromatography (HILIC). The majority of lipid analyses are conducted using reverse phase or HILIC chromatography (Cajka and Fiehn 2014).

Normal phase chromatography consists of a polar stationary phase and a nonpolar mobile phase. Molecules elute in order of increasing polarity. The retention of molecules decreases as the amount of polar solvent in the mobile phase increases. Reverse phase (RP) chromatography consists of hydrophobic stationary phase and a polar (aqueous) mobile phase. Hydrophilic compounds elute first. The more hydrophobic the molecule, the stronger it will bind to the stationary phase. Hydrophobic compounds are eluted by decreasing the polarity of the mobile phase using an organic (non-polar) solvent (Peterson and Cummings 2005). When developing this phospholipid method the testing of RP chromatography was insufficient to adequately separate phospholipid classes even when using formic acid as an additive. However, this issue was resolved by using HILIC chromatography.

Hydrophilic interaction chromatography (HILIC) combines the stationary phase typically used in normal phase chromatography with the mobile phase typically used in reverse phase chromatography. Analytes elute in the order of increasing polarity because more polar compounds have stronger interactions with the polar stationary phase (Onorato et al. 2010). Gradient elution usually begins with a high concentration of organic solvent which decreases over time while the percentage of aqueous solvent increases . Typically ionic additives such as ammonium formate are added to the mobile phase to control pH which contributes to the polarity of the analyte (Buszewski and Noga 2012). HILIC is useful for retention and separation of polar compounds and was shown to be the superior choice for phospholipid analysis when compared to RP chromatographic test runs. The HILIC column used for this lipidomics analysis is unbonded silica phase. This lipidomics method has been used to detect several classes of lipids but was developed for phospholipid separation and optimized based on eluting the head groups in order of increasing polarity.

All Ion Fragmentation

The ability to induce and detect all ion fragmentation (AIF) data is a feature of the Exactive plus orbitrap mass spectrometer. In AIF, packets of ions are trapped and sequentially stepped collision energies are applied to induce fragmentation (Gallart-Ayala et al. 2013). Full scan mass spectrometry data yields information on the parent masses of compounds. MS/MS parent/product data collected by triple quadrupole mass spectrometers yields information on the parent/product masses formed using specific collision energies. Full scan data doesn't rely on matching specific parent masses to product mass which allows for discovery based analysis while MS/MS data lacks any information on masses that were not pre-selected. AIF scans collect full scan data as well as fragmentation data (Geiger et al. 2010). When coupled with LC, retention times can be used to match parent masses with potential product masses. This allow for more accurate identification of compounds and yields the most data per scans. AIF is used for phospholipid detection to identify head group and tail fragments (Gallart-Ayala et al. 2013).

Phospholipidome of Candida albicans

Fungi of the genus *Candida* are opportunistic pathogens known to cause vulvovaginal, oral and invasive bloodstream infections in humans. Invasive infections are the most serious, with a mortality rate around 30% (Wisplinghoff et al. 2004; Morrell, Fraser and Kollef 2005). Currently, there are three main antifungal classes used to treat bloodstream infections of *Candida albicans*, the species that causes the majority of these infections (Pfaller et al. 2012). These therapies include azoles (e.g. fluconazole), echinocandins (e.g. caspofungin) and polyenes (e.g. amphotericin B). Unfortunately, these drugs have limited effectiveness due to documented cases of azole and echinocandin resistance (Mishra et al. 2007), the nephrotoxicity of amphotericin B (Holeman and Einstein 1963; Ghannoum and Rice 1999) and the requirement for intravenous administration of both amphotericin B and caspofungin. Furthermore, the recent rise in patients that are immunocompromised puts more people at risk every year (Low and Rotstein 2011), while also dramatically increasing healthcare costs (Mishra et al. 2007). As a result, it is of utmost importance to find novel antifungals to treat *C. albicans* potently and effectively.

Phospholipids are crucial components of biological membranes in both prokaryotes and eukaryotes. The phospholipidome of *C. albicans* is made up mostly of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG) and cardiolipin (CL) (Singh, Yadav and Prasad 2012). *Candida albicans* has a de novo method for producing phospholipids, which involves the conversion of cytidyldiphosphate-diacylglycerol (CDP-DAG) into PI, PG and PS (Fig. 1.1). Although PI, PG and PS can be end products, PG and PS can be further modified to form CL and PE, respectively, and PE can subsequently be methylated to produce PC. *Candida albicans* also utilizes exogenously provided ethanolamine and choline to produce PE and PC, respectively, via the Kennedy pathway (Gibellini and Smith 2010; Chen et al. 2010b; Henry, Kohlwein and Carman 2012).

Previous studies have identified the PS synthase (Cho1p) as a potential drug target in *C. albicans* (Braun et al. 2005; Chen et al. 2010b). Studies have been done on the enzymology and lipid profiles associated with PS synthase in Saccharomyces cerevisiae (Bae-Lee and Carman 1984), but little characterization of the orthologous enzyme or its effects on lipid profiles in *C. albicans* has been performed. Here we report the phospholipidome of the *C. albicans* cho1Δ/Δ and psd1Δ/Δ psd2Δ/Δ strains, as well as the enzyme kinetics of *C. albicans* Cho1p, finding both the Km and apparent Vmax to be in close agreement with those values reported for S. cerevisiae Cho1p (Bae-Lee and Carman 1984). The studies described in this report set the stage for further characterization of these enzymes as drug targets.

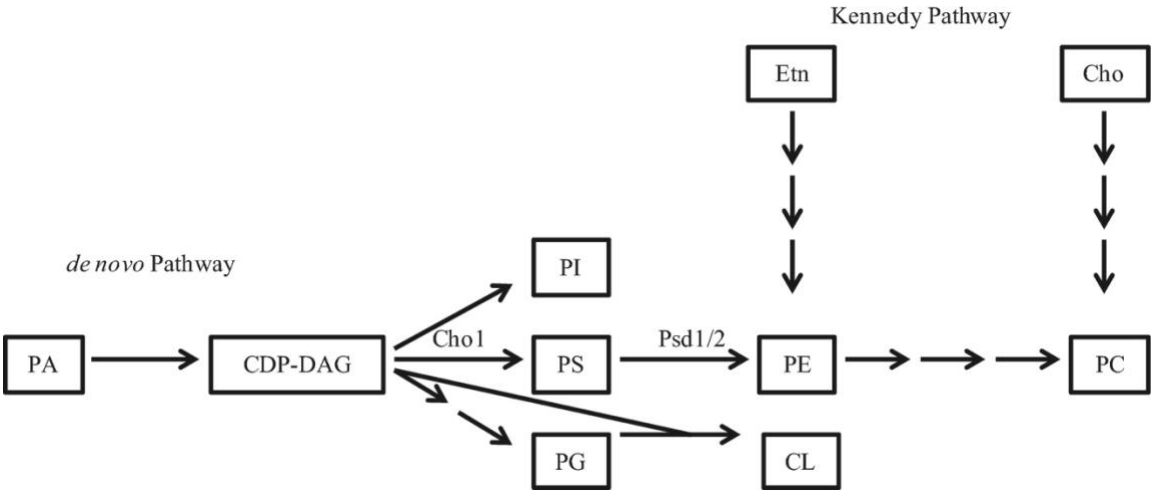

Figure 1.1: Phospholipid Biosynthesis Pathways in *C. albicans*

Candida albicans phospholipid biosynthesis occurs via both an endogenous pathway, the de novo pathway and an exogenous pathway, the Kennedy pathway. The precursors for producing the most common phospholipids are PA and CDP-DAG. CDP-DAG is then converted into PI, PS or PG. The endogenously produced PS can then be decarboxylated via Psd1 or Psd2 (Psd1/2) into PE and then further methylated into PC. In the Kennedy pathway, exogenous ethanolamine (Etn) and/or choline (Cho) are brought into the cell and converted into PE and PC. PG and CDP-DAG can be combined to generate CL. Abbreviations: PA, phosphatidic acid; CDP-DAG, cytidine diphosphate-diacylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; CL, cardiolipin; PC, phosphatidylcholine.

Materials and Methods

Strains Used

The SC5314 (wild-type) strain of *Candida albicans* and mutants used in this study have been previously described by Chen et al. (2010b). These include cho1Δ/Δ (YLC337), cho1Δ/Δ::CHO1 (YLC344), psd1Δ/Δ (YLC280), psd1Δ/Δ::PSD1 (YLC294), psd2Δ/Δ (YLC271), psd2Δ/Δ::PSD2 (YLC290) and psd1Δ/Δ psd2Δ/Δ (YLC375). The media used to culture strains was YPD (1% yeast extract, 2% peptone and 2% dextrose).

Lipid isolation for mass spectrometry analysis

Lipid isolations were adapted from the protocol of Singh et al. (2010). Cultures were grown overnight, shaking at 30°C in 5 mL of YPD. Cultures were then diluted to 0.4 OD600 in 25 mL of YPD and grown for 6 h, shaking at 30°C. After 6 h, cultures were pelleted and washed twice with PBS. The final pellets were incubated for at least 1 h at -80°C, and then lyophilized overnight. Dry mass was then recorded for normalization and pellets were suspended in 500 µL to 1 mL of PBS. The resulting thick suspension was then transferred to Teflon-capped glass tubes (Pyrex) and 1.5 mL of methanol was added. Two scoops of 150–212 µm sized glass beads (Sigma-Aldrich, St. Louis, MO, USA) were added to each tube. Cells were lysed by vigorous vortexing for 30 s punctuated by 30-s incubations on ice. Three mL of chloroform was added and the solution was vortexed briefly before being transferred to 15 mL glass funnel filtration system (Millipore, Billerica, MA, USA) with 24 mm glass microfiber filters (Whatman). Liquid collected was then poured into a separating funnel and washed with 900 µL of sterile 0.9% NaCl. The mixture was allowed to sit and separate for at least 5 min, or until adequate separation of the organic and aqueous layers was observed. The lower organic layer only was collected carefully into fresh Teflon-capped glass tubes. The organic layer was then dried under nitrogen gas until completely dry and stored at –20°C. Immediately before mass spectrometric analysis, the lipid extracts were resuspended in 300 µL of 9:1 methanol:chloroform (v/v).

Mass spectrometry lipidomics

Lipid extracts were separated on a Kinetex HILIC column (150 mm x 2.1 mm, 2.6 µm: (Phenomenex, Torrance, CA, USA) connected to an Ultimate 3000 UltraHigh Performance Liquid Chromatograph (UHPLC) with autosampler and an Exactive benchtop Orbitrap mass spectrometer (MS) (Thermo Fisher Scientific, San Jose, CA, USA) equipped with an electrospray ionization (ESI) probe. The column oven temperature was maintained at 25°C, and the temperature of the autosampler was set to 4°C. For each analysis, 10 µL was injected onto the column. Separations ran for 35 min at a UHPLC flow rate of 0.2 mL/min with mobile phases A and B consisting of 10 mM aqueous ammonium formate pH 3 and 10 mM ammonium formate pH 3 in 93% (v/v) respectively. The gradient started at 100% B and was altered based on the following profile: t = 0 min, 100% B; t = 1 min, 100% B; t = 15 min, 81% B, 29% A; t = 15.1 min, 48% B, 52% A; t = 25 min, 48% B, 52% A; t = 25.1 min, 100% B, t = 35 min, 100% B. The same LC conditions and buffers were used for all MS experiments described below.

The MS spray voltage was set to 4 kV, and the heated capillary temperature was set at 350°C. The sheath and auxiliary gas flow rates were set to 25 units and 10 units, respectively. These conditions were held constant for both positive and negative ionization mode acquisitions, which were both performed for every sample. External mass calibration was accomplished using the standard calibration mixture and protocol from the manufacturer approximately every 2 days. For full scan profiling experiments, the MS was run with resolution of 140 000 with a scan range of 113–1700 m/z. For lipid identification studies, HCD fragmentation experiments were run. These experiments were performed by alternating between full scan acquisitions and all ion fragmentation HCD scans. Samples were analyzed in both positive and negative mode, and full scan settings were the same as listed above. For the all ion fragmentation scans, the resolution was 140 000 with a scan range of 113–1700 m/z. The normalized collision energy was 30 eV, and a stepped collision energy algorithm of 50% was used. Full scan MS data were evaluated using Maven software (Melamud, Vastag and Rabinowitz 2010), and lipid classes were identified by their fragments using the Xcalibur software

package (Thermo Fisher Scientific) and information from the LIPID MAPS initiative (Fahy et al. 2007).

Lipid species were verified using retention times, high mass accuracy and fragmentation data. Internal standards were not used in this study; therefore, the relative amounts of each phospholipid species are presented. Interclass comparisons are possible, although some approximation of the relative intensities is inherent due to differing ionization efficiencies among lipids with different acyl chain lengths. Adequate separation of phospholipids was seen as shown in Fig. 1.6.

PS synthase assay

This procedure was done as previously described with minor alterations (Bae-Lee and Carman 1984; Matsuo et al. 2007; Cassilly et al. 2016). Cultures were grown overnight and then diluted into 1 L YPD, to ~0.1 OD600/mL. These cultures were shaken at 30°C for 6 to 10 h. Cells were then harvested by centrifugation at 6000 x g for 20 min. Pellets were then transferred to 50 mL conical tubes and washed with water and re-pelleted. Supernatant was removed and the wet weight of the samples was taken. Cell pellets were stored overnight in –80°C. The following day, a cold mixture of 0.1 M Tris-Cl pH 7.5, 5 mM β-mercaptoethanol (BME), 10% glycerol, and protease inhibitors (phenylmethylsuphonyl fluoride, leupeptin and pepstatin) was added to the frozen pellets (1mL/g [wet weight]) and allowed to thaw on ice. Cells were lysed using a either a French Press (three passes at ~13 000 lb/in2) or using osmotic lysis (Graham et al. 1994). The homogenate was centrifuged at 4°C for 5 min at 3000 rpm to clear unbroken cells and heavy material. Supernatant was then spun again at 27000× g for 10 min at 4°C. For some experiments, the resulting supernatant was then spun at 100 000× g to collect the lower density membranes. Pellets were resuspended in 500 µL to 1 mL of 0.1 M Tris-Cl pH 7.5, 5 mM BME, 10% glycerol and protease inhibitors. This mixture was aliquoted into microcentrifuge tubes and homogenized to break apart clumps, keeping on ice as much as possible. Total crude protein concentration was determined using a Bradford assay. The optimal assay mixture contained 50 mM Tris-HCl pH 7.5,

0.1% Triton X-100, 0.5 mM MnCl2 and 0.1 mM CDP-DAG (Avanti Polar Lipids, Alabaster, AL, USA) added as a suspension in 1% Triton X-100 and 0.4–0.5 mg protein in a total volume of 0.1 mL.

The PS synthase assay was performed by monitoring the incorporation of 0.5 mM L-serine spiked with 5% (by volume) [3H]-L-serine (~20 Ci/mmol) into the chloroform-soluble product at 37°C for a predetermined amount of time. The reaction was terminated by the addition of 1 mL chloroform: methanol (2:1). Following a low-speed spin, 800 to 1000 µL of the supernatant was removed to a fresh tube and washed with 200 µL 0.9% NaCl. Following a second low-speed spin, 400–500 µL of the chloroform phase was removed to a new tube and washed with 500 µL of chloroform: methanol: 0.9% NaCl (3:48:47). Following a third low-speed spin, 200–300 µL was transferred into scintillation vials (Thermo Fisher Scientific). Tubes were left open in the hood until dried fully. The next day, 2.5 mL scintillation fluid was added to each tube and run through the scintillation counter.

Statistical analysis

Graphs were made using GraphPad Prism version 6.04. Unpaired t-tests were used to determine significance between results. The lipidomics data were normalized by dry weight prior to statistical analyses.

Results and Discussion

Phospholipids synthesized in the de novo pathway are dominated by 34:n species

Phospholipid profiles have previously been generated for the cho1Δ/Δ, psd1Δ/Δ and psd1Δ/Δ psd2Δ/Δ mutants using [32P]-labeled phospholipids and thin layer chromatography (TLC) (Chen et al. 2010b). However, TLC only reveals levels of different lipid classes (polar head groups). We also wanted to determine the differences among the individual species (fatty acid composition) within each class. Thus, profiles of

the major phospholipid classes were generated via lipidomics from lipids extracted from wild-type, cho1Δ/Δ, cho1Δ/Δ::CHO1, psd1Δ/Δ, psd1Δ/Δ::PSD1, psd2Δ/Δ, psd2Δ/Δ::PSD2 and psd1Δ/Δ psd2Δ/Δ strains of *Candida albicans*. The lipids were analyzed by UHPLC-ESI-MS using the protocol described in methods and materials and adequate separation of standard phospholipids was observed (Fig. 1.6). Profiles of PA, CDP-DAG, PI, PG, CL, PS, PE and PC from three biological replicates were generated and are shown in Fig. 1.2. Statistically significant differences for particular species within each class compared to wild-type are shown in Table 1.1 (found in appendix).

In the cho1Δ/Δ mutant, which lacks PS synthase (the initial step in the de novo pathway, Fig. 1.1), PS is essentially absent, as expected (Chen et al. 2010a) (Fig. 1.2). The cho1Δ/Δ::CHO1 reintegrant strain exhibited a modest return of PS levels as compared with the cho1Δ/Δ and wild-type strains. The reintegrant strain has only one copy of CHO1, so haploinsufficiency is a possible explanation of this result (Uhl et al. 2003). However, since the level of PS in the reintegrant is only about 37% of the wild type (Fig. 1.2), one allele may be dominant over the other. *Candida albicans* has well-documented heterozygosity, which could account for this result (Eckert and Muhlschlegel 2009). We were concerned that the lower level of PS synthase activity could be the product of a defect in the reintegration construct, but independently generated reintegration constructs yielded similar results in the PS synthase assay (data not shown).

Figure 1.2: Phospholipid Species Profiles

The phospholipid species profiles for the major classes of phospholipids in *C. albicans* phospholipid synthesis mutants. (A) The phospholipid species profiles are shown for the major phospholipid classes of phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylinositol (PI) and cardiolipin (CL). (B) The species profiles detected are shown for the precursor lipids phosphatidic acid (PA) and cytidyldiphosphate-diacylglycerol (CDP-DAG). For each lipid class, a stacked bar graph is used and the species associated with each color is shown in the legend based on the combined carbon content and number of unsaturated bonds within the fatty acid component of the phospholipid. The Y-axis of the graph is the area under the peak for each species based on spectral profiles. The X-axis is the strains, which are as follows: wild-type (WT), cho1 (cho1Δ/Δ), psd1 (psd1Δ/Δ), psd1,2 (psd1Δ/Δ psd2Δ/Δ), psd2 (psd2Δ/Δ), psd2R (psd2Δ/Δ::PSD2), psd1R (psd1Δ/Δ::PSD1).

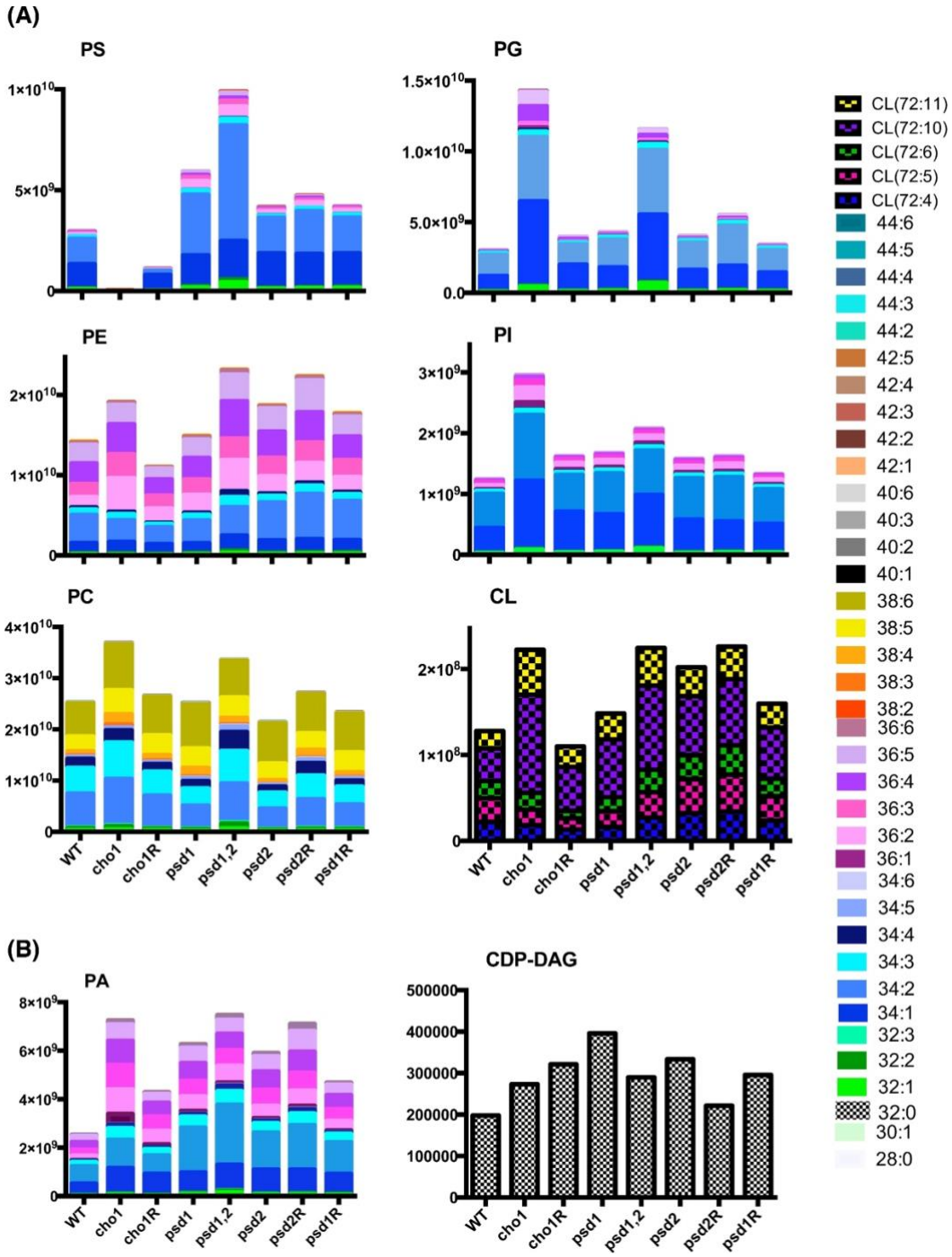

Figure 1.2: Continued

The distribution of PS species in the wild-type consists predominantly of 34:n species, with 34:2 being the most prevalent (Fig. 1.2). The psd1Δ/Δ mutant, which lacks the dominant PS decarboxylase (predicted to be localized to the mitochondria; Birner et al. 2001, 2003) that converts PS to PE, exhibits an overall increase in PS, but especially of the 34:2 species. A slight increase in PS is seen in the psd2Δ/Δ mutant, which lacks the alternative PS decarboxylase (predicted to localize to the Golgi/endosome; Trotter and Voelker 1995). The reintegrant strains psd1Δ/Δ::PSD1 and psd2Δ/Δ::PSD2 also show slight increases in levels of PS. The psd1Δ/Δ psd2Δ/Δ mutant, which lacks all PS decarboxylase activity (Chen et al. 2010a), has over a 3-fold increase in PS species overall, but individual species are overrepresented by 2- to 5-fold. The 34:n species, which were the most abundant in wild type, are also the most abundant in psd1Δ/Δ psd2Δ/Δ and are overrepresented by ~5 fold compared to wild type. However, it appears that all species are decarboxylated to some extent, given that they build up as well.

PE is the direct downstream product of PS decarboxylation (Fig. 1.1), and loss of PS was expected to result in a sizeable decrease in PE, but surprisingly we saw increases in PE levels in most of our mutants (Fig. 1.2). Most of these changes are not statistically significant in the majority of mutants, including cho1Δ/Δ (Table 1.1), although they are for psd1Δ/Δ psd2Δ/Δ. The maintenance of PE levels by the cho1Δ/Δ and psd1Δ/Δ psd2Δ/Δ mutants is likely due to growing these cultures in YPD, a rich medium containing ethanolamine and choline. Since the cho1Δ/Δ and psd1Δ/Δ psd2Δ/Δ mutants have no production of PE via the CDP-DAG pathway, it is likely that these mutants compensate through activity from the Kennedy pathway in order to synthesize PE. However, this remains to be experimentally verified. The psd1Δ/Δ mutant, which is the missing mitochondrial PS decarboxylase activity, showed wild-type levels of PE, suggesting that the loss of Psd1p was rescued by the redundant function of Psd2p. The psd2Δ/Δ strain exhibited an increase in PE, but this was again not statistically significant. The psd2Δ/Δ::PSD2 strain did have significant increases in many phospholipids, which was unexpected. The psd1Δ/Δ::PSD1 did not show many significant differences from wild type. Overall, the majority of PE species in wild type are

of 36:n or 34:n, and even in the psd1Δ/Δ psd2Δ/Δ where the changes were significant, this only shifted slightly compared to wild type.

Since the majority of PS is 34:n in wild-type cells, the Kennedy pathway may account for half of the PE population (34:n) or significant acyl remodeling of PS-derived PE is used to create other species after, or just prior to, decarboxylation. However, since a similar distribution of PE is found in wild-type, cho1Δ/Δ (totally lacks PS) and psd1Δ/Δ psd2Δ/Δ strains (cannot convert PS to PE), this suggests that most of the 36:n PE is derived from the Kennedy pathway, and the Kennedy pathway also synthesizes 34:n PE efficiently or converts 36:n by acyl remodeling.

Previous studies with Saccharomyces cerevisiae showed an accumulation of PC in the Sccho1Δ mutant based on TLC analysis (Atkinson, Fogel and Henry 1980). Indeed, our data show an ~1.5-fold increase in the level of PC in both the cho1Δ/Δ and psd1Δ/Δ psd2Δ/Δ mutants when compared to the wild type (Fig. 1.2). The most obvious common relationship between cho1Δ/Δ and psd1Δ/Δ psd2Δ/Δ is that both have a complete loss of de novo PE synthesis, which may indicate that this metabolic alteration causes an increase in PC synthesis via the Kennedy pathway. In the psd1Δ/Δ psd2Δ/Δ or cho1Δ/Δ mutants, PC can be synthesized directly from choline and DAG via the Kennedy pathway or by methylation of Kennedy-pathway-derived PE via the de novo pathway (Fig. 1.1). However, from this data it is not possible to determine the level of PC coming directly from Kennedy pathway versus the methylation of Kennedy-pathway-derived PE to PC. Interestingly, the cho1Δ/Δ::CHO1 strain, which has only one allele of CHO1, has wild-type levels of PC. Loss of PS decarboxylase activity in psd1Δ/Δ or psd2Δ/Δ mutants has also little effect on PC levels when compared with that of wild type. Although there is a slight decrease in PC in the psd2Δ/Δ mutant, it does not appear to be statistically significant. These findings suggest that the organism strictly controls the production of PC to at least maintain wild-type levels. The majority of PC species are 36:n and 38:n. It is possible that the 36:n PC is formed through the de novo methylation of 36:n PE. The 38:n species are probably produced from the importation of exogenous

choline. However, there are no striking changes within lipid species across mutant strains to support or falsify these hypotheses.

Increases in other CDP-DAG-derived phospholipids may be caused by an abundance of substrate in the PS synthase and PS decarboxylase mutants

Phosphatidic acid (PA) is the precursor for CDP-DAG (Henry, Kohlwein and Carman 2012) that is used to produce PS, PG and PI (Fig. 1.1). The cho1Δ/Δ mutant showed a nearly 3-fold increase in PA levels when compared to wild type (Fig. 1.2). These results were expected because the loss of PS synthesis causes a major blockage in de novo phospholipid synthesis, which would in turn lead to a build-up of the precursor molecule PA (substrate for CDP-DAG). This is further supported by the intermediate level of PA in cho1Δ/Δ::CHO1, which correlates with the partial return of PS production, and thus increased usage of PA. Varying increases are shown in PA levels within psd1Δ/Δ and psd2Δ/Δ mutants and their reintegrant strains, again potentially relating back to the build-up of PS in these strains, which translates into a build-up of PA. This is supported by the large increase in PA seen in psd1Δ/Δ psd2Δ/Δ, which has no de novo PE production, and thus represents a blockage in this biosynthetic pathway.

We also aimed to analyze the levels of CDP-DAG within our mutants and found that we could only detect 32:0 species and that the overall levels of this precursor lipid were extremely low (Fig. 1.2B). This suggests that there is a high turnover of CDP-DAG and that it is rapidly used to produce PS, PI or PG. Although there was some variability within the CDP-DAG levels, including increases in most of the mutants tested, none of these changes were statistically significant when compared to wild type. However, the change in CDP-DAG levels within our mutants again could easily be attributed to a back-up in the de novo synthesis (either a halt of PS production or usage) which causes increases in CDP-DAG levels.

In addition to the precursors, we also looked at the levels of the other two phospholipids produced from CDP-DAG, PI and PG, as well as CL, which is produced from PG (Henry, Kohlwein and Carman 2012). Both the cho1Δ/Δ and the psd1Δ/Δ psd2Δ/Δ

mutants show increases in PI and PG levels. Where the cho1Δ/Δ PI levels increase by around 2.5-fold, the psd1Δ/Δ psd2Δ/Δ increase is more modest. For PG we see an increase in both cho1Δ/Δ and psd1Δ/Δpsd2Δ/Δ, at ~5-fold and 4-fold, respectively. These findings could be a result of more CDP-DAG being available for the production of PI and PG due to a blockage of PS synthesis (cho1Δ/Δ) or a build-up in PS (psd1Δ/Δ psd2Δ/Δ). PI and PG seem to be tightly controlled, however, because in all other mutant and reintegrant strains the levels are closer to wild type. Finally, levels of CL correspond well with the changes in PG within our mutant strains. This indicates that some of the excess PG produced can be further modified to produce CL. These findings correlate well with studies in S. cerevisiae and confirm our previous results with TLC (Atkinson, Fogel and Henry 1980; Chen et al. 2010b). Interestingly, as with PS, the most abundant species of PI and PG appear to be 34:n; thus, it is possible that most CDP-DAG destined for PS synthesis is 34:n, but our lipidomics yields of CDP-DAG were too low to verify this, and it may be 34:n CDP-DAG is so rapidly processed into other lipids that it is hard to detect at steady state.

PS synthesis in PS synthase and PS decarboxylase mutants

The psd1Δ/Δ psd2Δ/Δ mutant exhibits a large increase in PS levels (Fig. 1.2), which is presumed to be caused by decreased decarboxylation of PS to PE. However, it is possible that this is due to increased PS synthase activity instead. This seemed doubtful, but to test this, an in vitro PS synthase assay was performed on cellular membranes, which were isolated from cell lysates via a 27 000 × g centrifugation step. Additional low-density membranes were isolated via a 100 000 × g centrifugation step. However, as highly variable results were found in the 100 000 × g membrane prep, data were generated from the 27 000 × g membranes which provided more consistent results. The assay was used to compare the incorporation of [3H]-serine into membranes on the addition of CDP-DAG. We compared incorporation for wild-type, cho1Δ/Δ, cho1Δ/Δ::CHO1, psd1Δ/Δ, psd1Δ/Δ psd2Δ/Δ and psd1Δ/Δ::PSD1 strains. Wild-type levels of PS synthase activity were seen in the wild-type, psd1Δ/Δ and psd1Δ/Δ psd2Δ/Δ strains, indicating that these strains have similar enzyme activities

(Fig. 1.3). Thus, the excess PS in the psd1Δ/Δ psd2Δ/Δ mutant is likely a product of the loss of PS decarboxylase activity and build-up of substrate, not excess PS synthase activity.

As expected from this assay, the cho1Δ/Δ mutant exhibited no PS synthase activity. However, the cho1Δ/Δ::CHO1 reintegrant strain had only about 10% of the activity of the wild type. This compares with the decreased PS levels in the reintegrant as measured by lipidomics (Fig. 1.2). Haploinsufficiency may account for this lower activity.

Candida albicans Cho1p enzyme kinetics are similar to those reported for Saccharomyces cerevisiae

The wild-type and other strains seemed to have similar PS synthase activity levels, but it was of interest to know more about the enzyme kinetics of *C. albicans* Cho1p, if it is to be considered for potential use as a drug target. The $K_m$and apparent V_{max} of this enzyme were calculated for both serine and CDP-DAG (Fig. 1.4). L-Serine, where the CDP-DAG was held constant at 0.1 mM, yielded a K_m of 1.2 ± 0.57 mM and an apparent V_{max} of 0.15 ± 0.02 nmole/min/mg of protein (Fig. 1.4A). For CDP-DAG, where the serine was held constant at 2.5 mM, a K_m of 43.17 ± 19.18 μM and an apparent V_{max} of 0.19 ± 0.023 nmole/min/mg of protein (Fig. 1.4B) were obtained. Interestingly, the intracellular concentration of serine in *S. cerevisiae* has been estimated to be around 2 mM on average (Hans, Heinzle and Wittmann 2003), which fits well with the K_m for serine. These assays were performed on the crude 27 000 × *g*membranes in order to determine their kinetics within native membranes. These compare relatively closely with what has been reported for *S. cerevisiae*(Bae-Lee and Carman 1984).

To control for the possibility that similar substrate molecules to serine might give a similar activity, which could cast doubt on the accuracy of the assay, we determined whether increasing concentrations of cold D-serine or threonine (similar amino acid) could compete with [3H]-L-serine for incorporation into [3H]-PS.

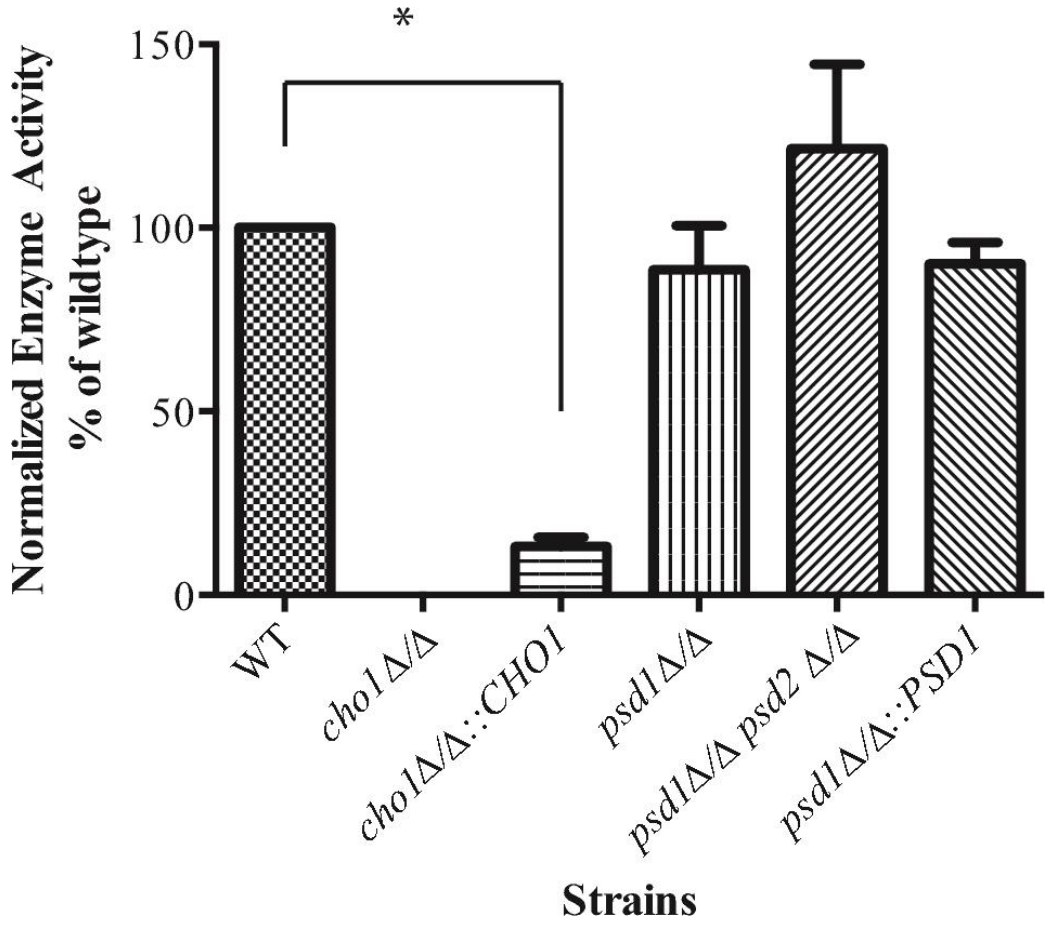

Figure 1.3: PS synthase activity across strains

Cho1p activity within different mutant strains of *C. albicans*. Cellular membranes containing Cho1p were isolated and treated with CDP-DAG and [^{3}H]-serine. Activity is measured as counts per minute per milligram of protein. Values are shown as an average of three experiments averaged and normalized to the wild-type (WT) control. $P < 0.0001$.

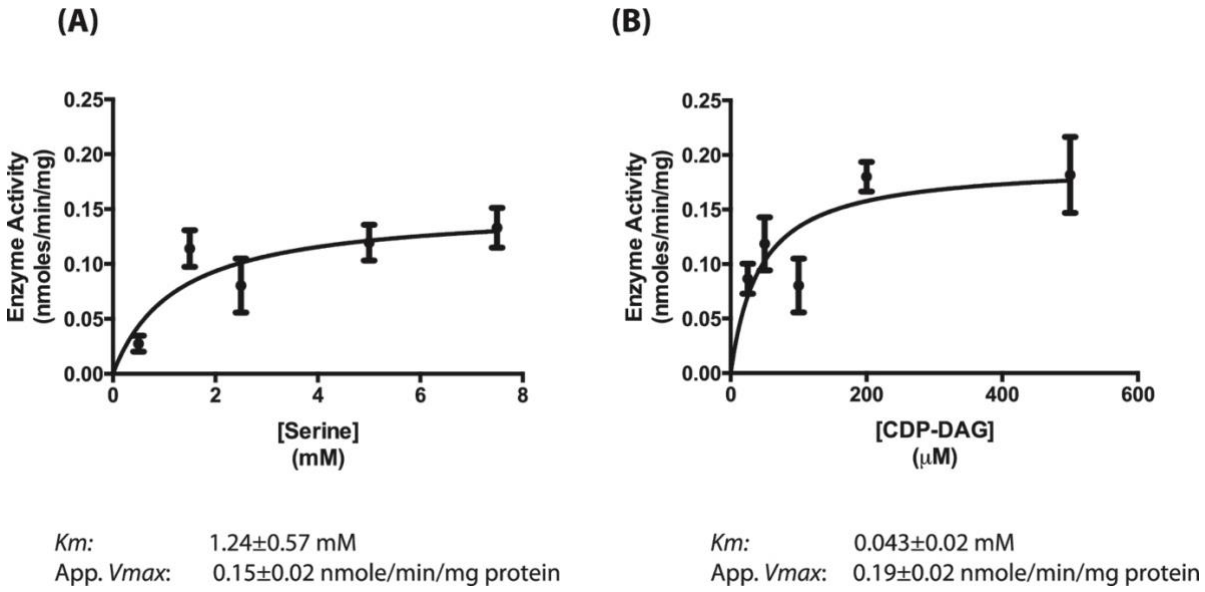

Figure 1.4: Enzyme kinetics for Cho1p

The PS synthase assay was performed with varying concentrations of substrate over time courses to generate enzyme kinetics for both substrates. (A) The Michaelis-Menton curve for the L-serine substrate shows a Km of 1.243 ± 0.5715 mM and an apparent Vmax of 0.1514 ± 0.02059 nmole/min/mg of protein. (B) The Michaelis-Menton curve for the CDP-DAG substrate shows a Km of 43.17 ± 19.18 μM and an apparent Vmax of 0.1919 ± 0.02361 nmole/min/mg of protein.

Only at the highest concentration of 200 mM (×400) did the cold D-serine show any competition with L-serine (Fig. 1.5). Threonine showed no statistically significant competition with L-serine, even at 200 mM (×400). As a control, cold L-serine eliminated incorporation of [3H]-L-serine at a ×10 concentration (5 mM). Thus, this assay would not yield a product with these two similar molecules, suggesting that the assay is specifically measuring activity against L-serine.

Conclusions

The loss of de novo PE synthesis and/or PS synthesis has striking effects on the phospholipidome, which are consistent with what we know from fungal phospholipid metabolism. In particular, the loss of the de novo pathway for PS and/or PE synthesis appears to increase the synthesis of other CDP-DAG-dependent phospholipids like PI and PG (Fig. 1.2). Surprisingly, PE and PC levels appear to be buffered against change, presumably by the activity of the Kennedy pathway. The 34:n species are the most prominent forms of PS, and 34:2 in particular, appears to be the most actively used substrate by PS decarboxylase enzymes to synthesize PE. The 34:2 species is also what builds up in PI and PG, which may suggest that either Cho1p preferentially uses 34:2 CDP-DAG or 34:2 CDP-DAG is what it encounters in its subdomains in the ER.

Finally, our analysis of the enzymology of Cho1p from *Candida albicans* reveals that it is very similar to Saccharomyces cerevisiae, and changes in PS levels follow changes in Cho1p activity. For example, when PS synthesis activity is compared to PS levels in the wild-type, cho1Δ/Δ::CHO1 and cho1Δ/Δ strains, these measurements seem to correlate with one another rather closely (Figs 1.2 and 1.3). These analyses set the stage for better understanding how strategies to inhibit de novo PS or PE synthesis, which are required for virulence of *C. albicans* (Chen et al. 2010a), may impact overall lipid profiles and ability to cause disease.

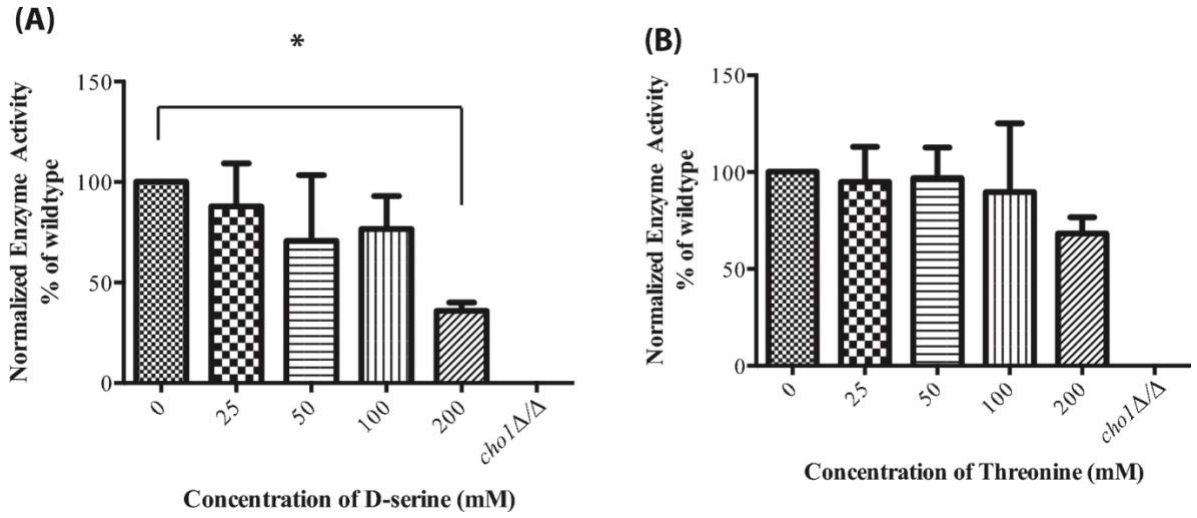

Figure 1.5: Cho1p shows specificity for L-serine

The PS synthase assay was performed with 0.5 mM [3H]-serine and 0.1 mM CDP-DAG. An excess of D-serine (A) or L-threonine (B) was added at varying concentrations. Only D-serine or L-threonine at concentrations 400-fold that of L-serine show any inhibition in [3H]-PS production. Values are shown as an average of three experiments averaged and normalized to the wild-type control. P = 0.0361.

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Appendix

All P values are relative to wild-type: **P value <0.01, *P value <0.05, the symbol (-) indicates the value is not significantly different from wild-type

Table 1.1: Statistical analysis of differences between species of lipids

Phosphatidylserine (PS)							
	cho1	cho1R	psd1	psd1,2	psd2	psd2R	psd1R
PS(28:0)	**	**	**	**	**	-	*
PS(30:1)	**	**	-	**	-	-	-
PS(32:1)	**	**	**	**	-	*	*
PS(32:2)	**	**	**	**	-	*	*
PS(34:1)	**	*	-	*	*	-	-
PS(34:2)	**	**	**	**	-	**	-
PS(34:3)	**	**	**	**	-	-	-
PS(34:4)	**	**	**	**	-	-	-
PS(34:5)	*	*	**	**	-	-	-
PS(36:2)	**	**	**	**	**	**	*
PS(36:3)	**	**	**	**	*	**	-
PS(36:4)	**	**	**	**	*	-	-
PS(36:5)	**	**	**	**	-	-	-
PS(36:6)	**	**	**	**	-	-	-
PS(38:1)	**	-	-	*	-	-	-
PS(38:2)	**	-	-	*	-	-	*
PS(38:3)	*	-	-	*	-	-	-

Phosphatidylethanolamine (PE)							
	cho1	cho1R	psd1	psd1,2	psd2	psd2R	psd1R
PE(32:1)	-	-	-	**	-	**	-
PE(32:2)	-	*	-	**	-	**	-
PE(32:3)	-	**	-	**	-	*	-
PE(34:1)	-	-	-	**	-	-	-

Table 1.1: Continued

Phosphatidylethanolamine (PE)							
	cho1	cho1R	psd1	psd1,2	psd2	psd2R	psd1R
PE(34:2)	-	*	-	-	-	**	-
PE(34:3)	-	-	-	**	-	**	-
PE(34:4)	-	-	-	**	-	**	-
PE(34:5)	-	**	-	**	-	**	-
PE(36:2)	*	*	-	**	**	**	-
PE(36:3)	*	-	-	**	*	**	-
PE(36:4)	-	-	-	**	-	**	-
PE(36:5)	-	-	-	*	-	**	-
PE(36:6)	-	-	-	**	-	**	-
PE(38:3)	*	*	-	-	-	-	-
PE(38:4)	**	*	-	-	-	-	-
PE(38:5)	**	**	-	*	-	-	-

Phosphatidylcholine (PC)							
	cho1	cho1R	psd1	psd1,2	psd2	psd2R	psd1R
PC(32:1)	-	-	-	**	-	-	-
PC(32:2)	-	-	-	**	-	-	-
PC(32:3)	-	-	-	-	-	-	-
PC(34:2)		-	-	-	-	-	-
PC(34:3)	-	-	-	-	-	-	-
PC(34:4)	-	-	-	*	-	-	-
PC(34:5)	-	-	*	*	-	*	-
PC(34:6)	-	-	**	-	-	*	-
PC(38:2)	**	-	-	**	-	-	-
PC(38:3)	-	-	-	-	-	-	-
PC(38:4)	**	-	**	-	-	-	-
PC(38:5)	-	-	-	-	-	-	-
PC(38:6)	-	-	-	-	-	-	-
PC(40:1)	-	-	-	-	-	-	-
PC(40:2)	-	-	*	-	-	-	-

Table 1.1: Continued

Phosphatidylcholine (PC)							
	cho1	cho1R	psd1	psd1,2	psd2	psd2R	psd1R
PC(40:3)	-	-	-	-	-	-	-
PC(40:6)	*	-	*	-	-	-	-
PC(42:1)	-	-	**	-	-	-	-
PC(42:2)	-	-	**	-	-	-	-
PC(42:3)	-	-	**	-	-	-	-
PC(42:4)	-	-	-	-	-	-	-
PC(42:5)	*	-	-	-	-	-	-
PC(44:2)	-	-	**	-	-	-	-
PC(44:3)	*	*	**	-	-	*	-
PC(44:4)	*	-	-	*	-	-	-
PC(44:5)	-	-	-	-	-	-	-
PC(44:6)	-	-	-	-	-	*	-

Phosphatidylglycerol (PG)							
	cho1	cho1R	psd1	psd1,2	psd2	psd2R	psd1R
PG(32:1)	**	-	-	**	-	**	-
PG(32:2)	*	-	-	**	-	-	-
PG(34:1)	**	*	-	**	*	**	-
PG(34:2)	*	-	-	**	-	**	-
PG(34:3)	*	-	-	**	-	**	-
PG(34:4)	*	-	-	**	*	*	-
PG(36:1)	*	-	*	*	-	**	-
PG(36:2)	*	-	-	**	-	**	-
PG(36:4)	*	-	-	**	-	**	-
PG(36:5)	*	-	-	**	-	**	-
PG(36:6)	-	-	-	*	-	*	-

Table 1.1: Continued

Phosphatidylinositol (PI)							
	cho1	cho1R	psd1	psd1,2	psd2	psd2R	psd1R
PI(32:2)	*	-	-	**	-	-	-
PI(32:1)	**	-	*	**	-	-	-
PI(34:4)	*	-	-	**	-	-	-
PI(34:3)	-	-	-	-	-	-	-
PI(34:2)	**	-	-	-	-	-	-
PI(34:1)	**	*	*	**	-	-	-
PI(36:6)	-	-	-	*	-	-	**
PI(36:5)	-	-	-	-	-	-	-
PI(36:4)	*	-	-	-	-	-	-
PI(36:3)	**	-	-	-	-	-	-
PI(36:2)	*	-	*	*	*	*	-
PI(36:1)	*	**	**	**	**	**	-

Cardiolipin (CL)							
	cho1	cho1R	psd1	psd1,2	psd2	psd2R	psd1R
CL(72:4)	-	-	-	-	-	-	-
CL(72:5)	-	*	-	-	-	-	-
CL(72:6)	-	**	-	-	-	-	-
CL(72:10)	*	-	**	**	**	*	-
CL(72:11)	*	-	*	*	**	*	-

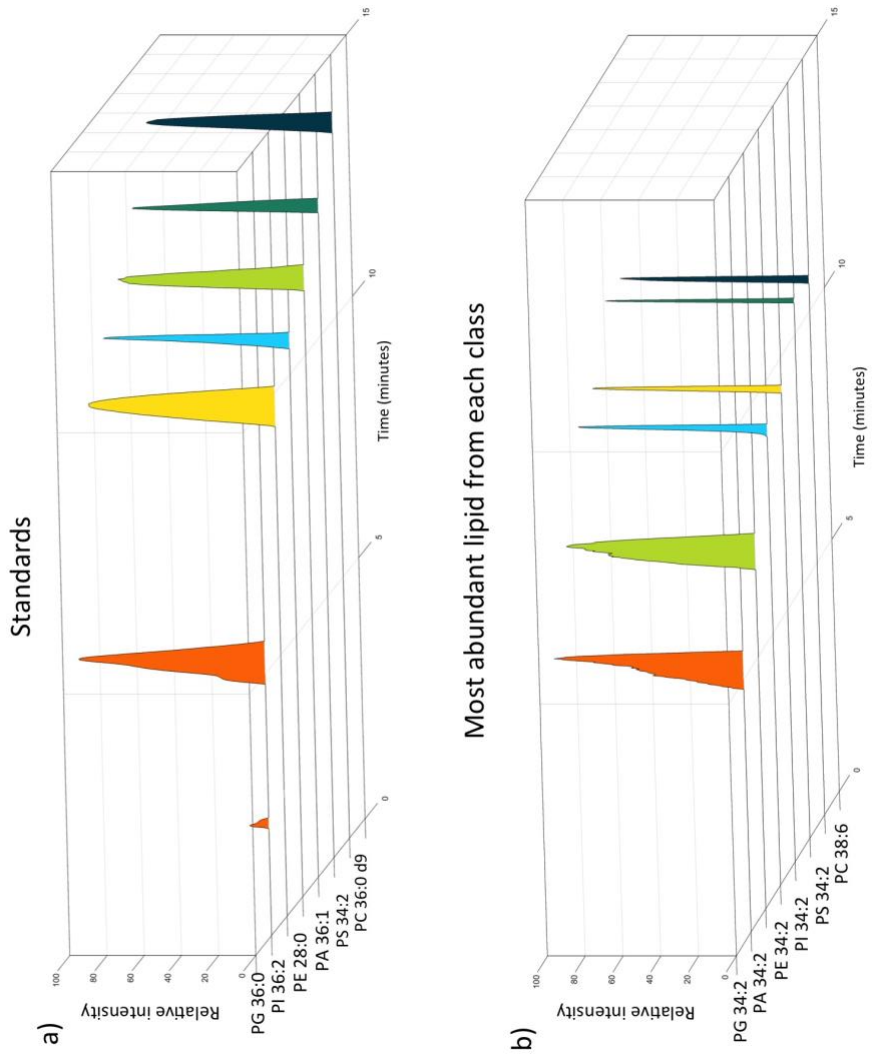

Figure 1.6: Stacked ion chromatograms

A) Stacked ion chromatograms from 0-15 minutes for standards suspended in 9:1 Methanol:Chloroform

B) Stacked ion chromatograms from 0-15 minutes for the most abundant lipids from each class from the wild type sample. Due to matrix effects from the extraction procedure the elution order is altered.

CHAPTER 2
METABOLIC FLUX OF CORAL INFECTED WITH BLACK BAND DISEASE

This chapter contains material expected to be part of publications by:
Farmer, A. T.; May, A. L.; Steen, A.; Richardson, L.; Campagna, S. R., Metabolic flux of coral infected with black band disease. *Coral Reefs* **2018**
(manuscript in final preparation)

Farmer, A. T.; Steen, A, FluxR: an R package to analyze isotopic flux. *BMC Research Notes* **2018**
(manuscript in final preparation)

ALM designed the experiment and collected the samples. ATF prepared the samples, analyzed the data, and wrote the paper. AS wrote the R package.

Abstract

Coral diseases are a major cause of coral death and black band disease (BBD) is considered the most virulent of coral diseases. BBD is identified by the distinct dark band that forms across the coral and causes tissue necrosis, leaving behind exposed coral skeleton. The differences between the microbial communities of healthy coral and diseased coral are reflected in the composition of metabolites. Mass spectrometry-based metabolomics was used to identify and compare what metabolites were present and how their abundances differed between healthy and diseased coral to probe what role they may play in the progression of the disease.

Metabolomic flux experiments were performed in parallel, allowing us to study the movement of small molecules through metabolic pathways to elucidate the metabolic pathways that BBD is affecting. In this experiment, metabolites from the surface mucopolysaccharide layer (SML) of healthy coral were compared to metabolites from both the coral infected with BBD and from the black band itself. Flux experiments were conducted by spiking isotopically labeled acetate, glucose, and ammonium into environmental samples. An R package was developed to visualize and calculate the rate of flux.

The flux data show that there are differences in the uptake of glucose, acetate, and ammonium depending on the area of the coral from which the samples were taken. Samples taken from the black band show a higher turnover rate compared to samples taken from healthy parts of the coral. This analysis reveals higher metabolic activity during active infection and provides new insights into BBD progression.

Introduction

Black band disease

Worldwide coral reefs are dying, and the number of healthy coral reefs is rapidly declining. Coral reefs represent some of the most bio diverse environments on the planet. The death of a coral reef detrimentally affects all of the organisms that the reef harbors and that surround the reef. Thus, in aggregate, declining health and death of coral reefs impacts the entire ocean's ecosystem. Some causes of coral reef death include increased ocean temperature, increased ocean acidity, and diseases.[1] Coral diseases are a major cause of coral death. Black band disease (BBD) is considered the most virulent of coral diseases. BBD is identified by the distinct dark band that forms across the coral and causes tissue necrosis, leaving behind exposed coral skeleton.[2]

BBD is a consortium of microorganisms, consisting of cyanobacteria, sulfate-reducing bacteria, sulfide-oxidizing bacteria, non-photosynthetic bacteria, and marine fungi. The bacteria found in the black band are more abundant and diverse than the bacteria found on the same coral in a healthy region. Bacterial sequencing studies have shown the differences in bacteria communities between healthy coral, diseased coral, and between different parts of the diseased coral on the same reef.[3] These differences in the microbial community are reflected in the composition of metabolites.

Metabolomics refers to the analysis of the metabolome, which is the totality of metabolites in a cell. The small molecules, or metabolites, of a cell tell us about the final state of a cell and its environmental conditions following gene expression and post-translational modification. The final state of a cell is affected by changes to its

environment or genome.[4] The presence and amounts of metabolites will be different based on differences in the bacteria. By analyzing the metabolite composition, we hope to gain new insights into metabolic pathways and functions.

Prior studies have looked at identifying the types of bacteria.[5] While studying this disease at the gene level tells us what microorganisms are present, it will not tell us everything. Because of this, we must look at the composition of small molecules to see what's actually happening in the cells. We will use mass spectrometry-based metabolomics to identify what metabolites are present and how their abundance differs between healthy and diseased coral to probe what role they may play in the progression of the disease.

In this experiment, metabolites from the surface mucopolysaccharide layer (SML) of healthy coral were compared to metabolites from coral infected with BBD. To begin, samples were collected off the coast of Curaçao to investigate the metabolism of large reef building corals affected by BBD. The three sample types are BBD, HBBD, and SML. BBD samples were collected from the black band on diseased coral. HBBD samples were collected from healthy parts of coral that were infected with black band disease. SML samples were collected from healthy coral that was not infected with black band disease. Then, flux experiments were conducted by spiking isotope labeled acetate, glucose, and ammonium into environment samples, in order to probe the metabolic routes and calculate the rates that metabolites are being produced and used. ^{13}C acetate was chosen as a substrate to investigate metabolite flux through the TCA cycle, ^{13}C glucose was chose to investigate glycolysis, and ^{15}N ammonium was chosen to investigate nitrogen assimilation.

Metabolic flux

Metabolomic flux experiments aim to study the movement of small molecules through metabolic pathways.[6] Metabolite concentration studies are useful but can't reflect if changes in concentrations are due to decreased production or increased consumption.[7]

Using the assumption that cells grown in media are at steady state, we can use isotopically labelled substrates to calculate relative rates.[8] Flux calculations are based on the incorporation of isotopically labeled nutrients into downstream metabolic products. Theoretically larger fluxes are associated with a faster turnover of downstream metabolites from the isotopically labelled precursors.[9]

Data from flux experiments can help identify what pathways and what steps in these pathways are being affected by changes to the cells environment—such as temperature or disease—and thus, help elucidate the metabolic pathways that BBD is affecting. Comparing metabolic fluxes between healthy and diseased coral provides information on how BBD is affecting coral reef death.

Materials and Methods

Sample Collection

Samples were collected of the coast of Curaçao. Coral samples were taken from infected regions (BBD), healthy regions of infected coral (HBBD), and from the surface mucopolysaccharide layer of uninfected coral (SML). For the flux experiment triplicate samples were filtered and the filters placed in isotopically labeled glucose, acetate, or ammonium and the filters were then flash frozen with liquid nitrogen after 0, 25, 50, 75, 100, and 125 minutes. The filters were stored at -80°C.

Metabolomics Analysis

Samples were kept at -80˚C until extraction. The filters were extracted for water soluble metabolites with an extraction protocol to prevent the metabolome from changing once the extraction started.[10] The extraction solvent was 40:40:20 ACN:MeOH:H2O with 0.1% formic acid and was kept at 4˚C. The frozen filter was transferred to an empty petri dish and 1.3 mL of extraction solvent was pipetted onto the filter. The filter was unfolded using sterile forceps and set cell side down in the extraction solvent. Then the filter was left to extract for 15 minutes at -20˚C. After extracting, the solvent was transferred to an

eppendorf. The cells were then washed from the filter with 300 µL of extraction solvent and the solvent transferred to the same eppendorf. The sample was then centrifuged at 13,200 rpm for 5 minutes at 4 ˚C. After centrifugation, the supernatant was transferred to a vial for drying under N_2. The pellet was re-extracted with 50 µL of extraction solvent and left to extract for 15 minutes at -20 ˚C. Following extraction, the sample was centrifuged at 13,200 rpm for 5 minutes at 4 ˚C. The supernatant was transferred to the drying vial and the pellet extracted again with 50 µL of the extraction solvent. The sample was left to extract for 15 minutes at -20 ˚C and centrifuged again at 13,200 rpm for 5 minutes at 4 ˚C. The supernatant was transferred to the drying vial to dry under N_2. Once the samples were dry, they were then resuspended with 300 µL of water and placed in an autosampler vial for analysis.

Samples were placed in an autosampler tray kept at 4 ˚C. A 10 µL aliquot was injected through a Synergi 2.5-micron Hydro-RP 100, 100 x 2.00 mm LC column (Phenomenex) kept at 25 ˚C. The mass spectrometer was run in full scan mode with negative ionization using a method adapted from Rabinowitz.[11] The eluent was introduced into the MS via an electrospray ionization source conjoined to a Thermo Scientific Exactive Plus Orbitrap mass spectrometer through a 0.1 mm internal diameter fused silica capillary tube. The samples were run with a spray voltage of 3 kV. The nitrogen sheath gas was set to a flow rate of 10 psi with a capillary temperature of 320 ˚C. The AGC target was set to 3e6. The samples were analyzed with a resolution of 140,000 and a scan window of 85 to 800 *m/z* for from 0 to 9 minutes and 110 to 1000 m/z from 9 to 25 minutes. Solvent A consisted of 97:3—water to methanol—10 mM tributylamine, and 15 mM acetic acid. Solvent B was methanol. The gradient from 0 to 5 minutes was 0% B, from 5 to 13 minutes is 20% B, from 13 to 15.5 minutes is 55% B, from 15.5 to 19 minutes is 95% B, and from 19 to 25 minutes is 0% B with a flow rate of 200 µL/min.

The mass spectrometric flux data was processed using MAVEN[12] and matched to a library of metabolite standards based on high resolution *m/z* values and retention times. The MAVEN package integrates the area under the peak, and peak areas are used to compare metabolites.

Flux Analysis

An R package[13] was developed to calculate flux rates and create graphs showing isotope incorporation. Flux graphs were made based on the following equation where A_0 is the initial amount of ^{12}C (or ^{15}N) in the metabolite, A is the amount of ^{12}C (or ^{15}N) left after time t and k is the rate of decay.

$$A = A_0 e^{kt}$$

The flux R script used also calculated the statistical error associated with calculating the flux rates.

Results

The flux data show that there are differences in the uptake of glucose, acetate, and ammonium depending on the area of the coral from which the sample was taken. Samples taken from the black band show a higher flux rate than samples taken from healthy parts of the coral.

The time zero data was treated as a pool size experiment. The metabolite composition of each cell type was compared at time zero, prior to any isotopically labeled metabolite incorporation. The data was normalized using an internal ratio normalization method to account for differences in biomass.[14,15] The heat maps in figures 2.1, 2.2, and 2.3 show fold changes for metabolites between sample types for each experiment. The p-values were calculated using a student's t-test. At time zero, there are few differences in the metabolome between HBBD samples and SML samples. However, there are many differences in the metabolome between BBD compared to HBBD and SML.

Small molecules that were identified were investigated to see which metabolic pathways they are involved in.

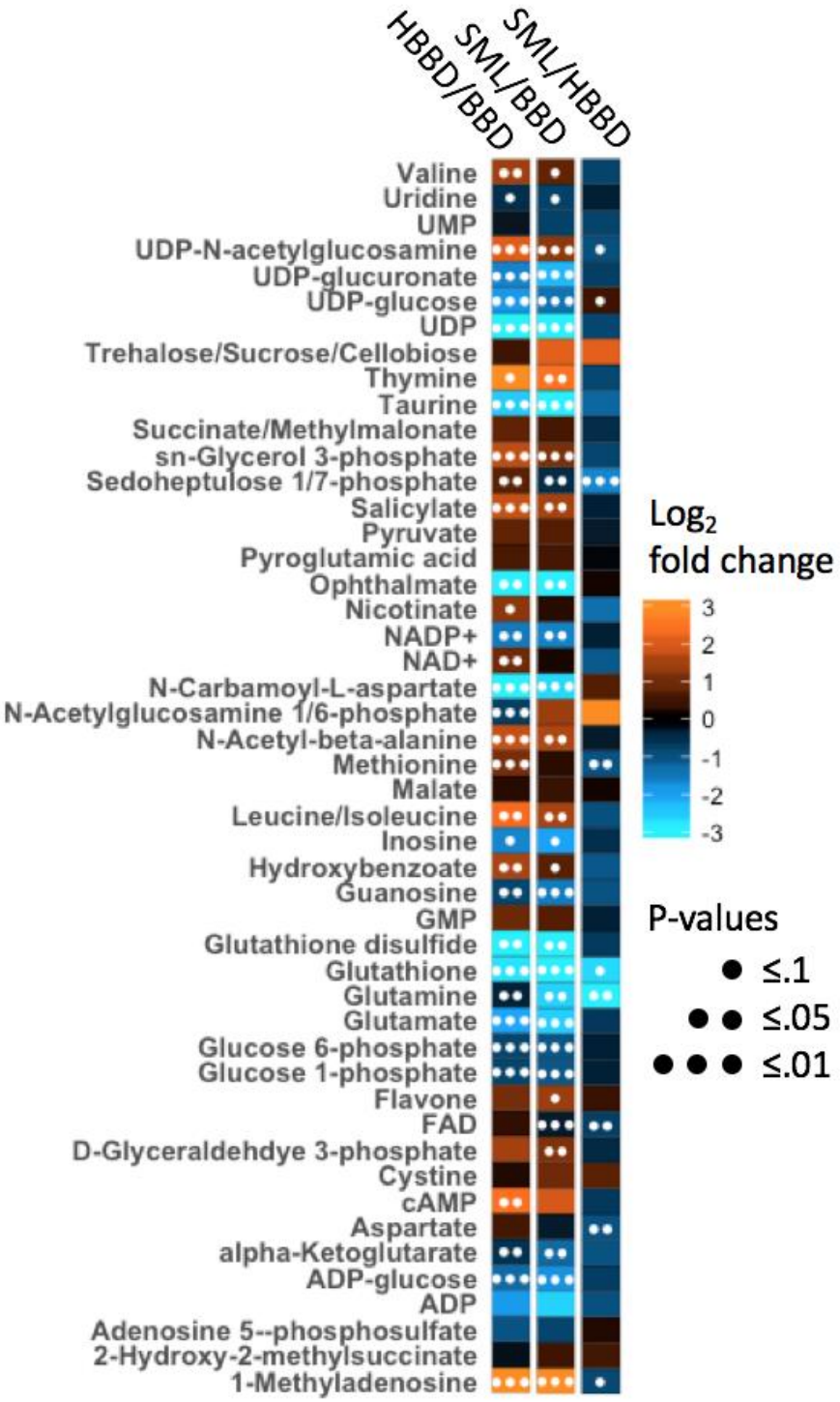

Figure 2.1: Time Zero Heatmap for Ammonium Experiment

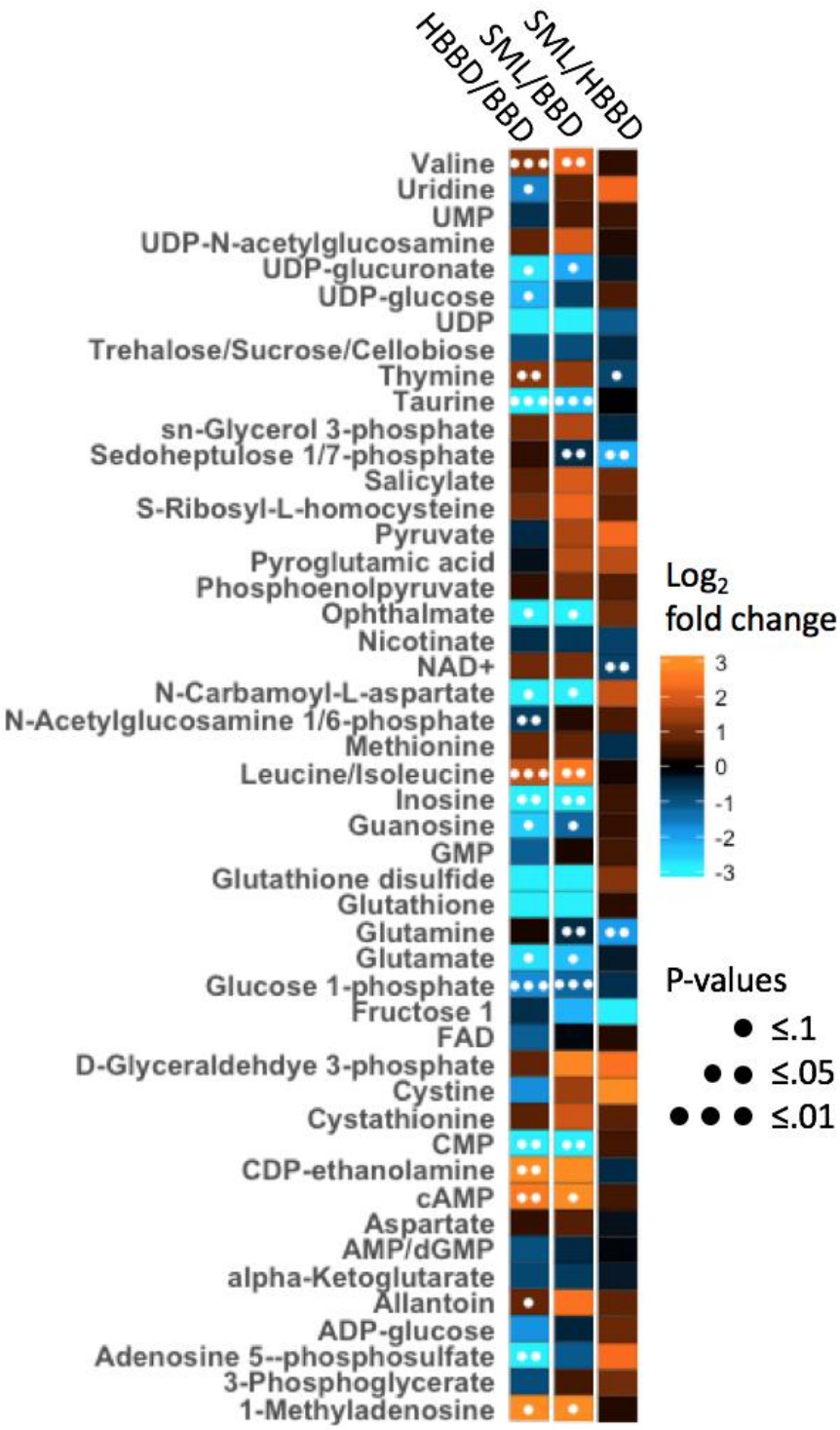

Figure 2.2: Time Zero Heatmap for Glucose Experiment

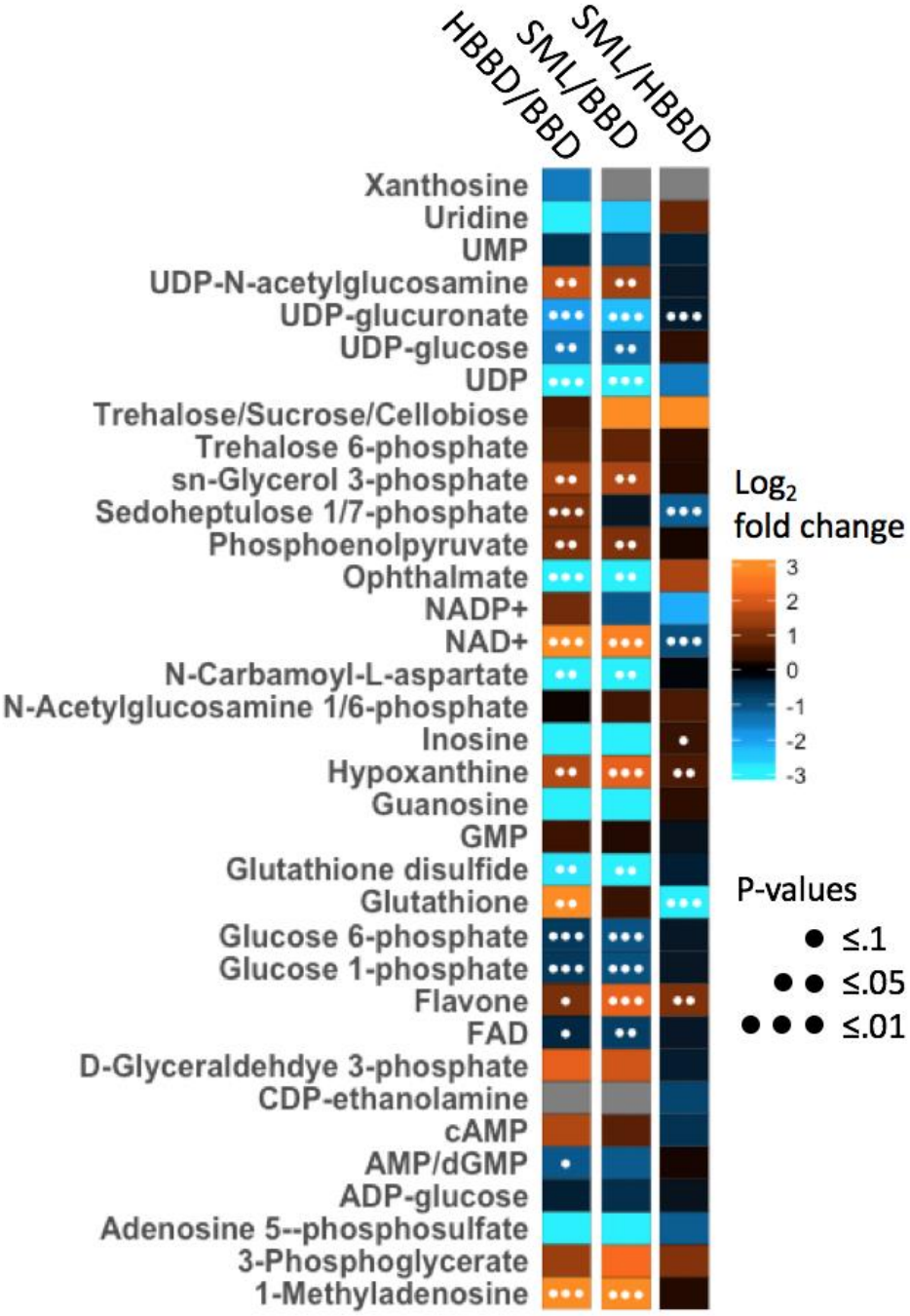

Figure 2.3: Time Zero Heatmap for Acetate Experiment

35 metabolites were detected in the time zero data in the acetate flux experiment: 8 metabolites in purine metabolism; 1 metabolite in carbon fixation; 1 metabolite in the TCA cycle; 2 metabolites in glutathione metabolism; 5 metabolites in glycolysis; 4 metabolites in pyrimidine metabolism.

48 metabolites were detected in the time zero data in the glucose flux experiment: 4 in purine metabolism; 8 metabolites in pyrimidine metabolism; 2 metabolites in carbon fixation; 1 metabolite in the TCA cycle; 3 metabolites involved in glutathione metabolism; 6 metabolites involved in glycolysis.

48 metabolites were detected in the time zero data in the ammonium flux experiment: 7 metabolites in the purine metabolism; 4 metabolites involved in glycolysis; 4 metabolites in glutathione metabolism; 6 metabolites in the pyrimidine metabolism; 1 metabolite in carbon fixation; 3 metabolites in the TCA cycle.

The flux data from the three different experiments were analyzed and the metabolites showing isotopic incorporation were identified. 41 metabolites showed incorporation in the glucose experiment. 35 metabolites showed incorporation in the acetate experiment. 22 metabolites showed incorporation in the ammonium experiment. These metabolites were investigated further, with the metabolites that showed significant incorporation identified as metabolites of interest. For the glucose flux, there were 4 metabolites of interest: UDP-D-glucuronate, glutamate, D-glyceraldehyde-3-phosphate, and FAD. For acetate, there were 8 metabolites of interest: aspartate, succinate, malate, UDP-D-glucose, glutathione disulfide, UDP-D-gluconarate, dimethylglycine, and di-hydroxy-acetone-phosphate. For ammonium there were 6 metabolites of interest: glutamate, glutamine, methionine, UDP-D-glucose, UDP-N-acetyl-glucosamine, and UDP-D-gluconarate. Flux graphs were created to visualize the metabolites that showed the most isotope incorporation and highest rate of flux.

Discussion

Ammonium Flux

Selected flux graphs for the ammonium experiment are shown in figure 2.4 with the corresponding fits in table 2.1. They show a decrease in isotope labeling for BBD in glutamine as compared to HBBD and SML. This indicates that the bacteria are not storing nitrogen in glutamine. Thus, either the bacterial consortium in BBD is using too much nitrogen to store or there is an excess of available nitrogen so it's not energy efficient to store. Previous studies have shown environmentally available nitrogen compounds increase virulence. Richardson et al. found that cyanobacteria isolated from BBD were unable to fix nitrogen; therefore, an increase in nitrogen availability will increase virulence.[16] According to Wittenberg et al. nutrient enrichment reduces coral health and can reduce its ability to fight off infection.[17] Previous studies have found that the black band is dominated by cyanobacteria and sulfide-oxidizing bacteria.[18] Kuta et al. found the increased nitrate concentration may be a result of the BBD consortium itself and its incomplete reduction of nitrate.[19]

The ammonium experiment also revealed an increase in the isotope labeling of UDP-glucuronate and UDP-glucose for BBD samples compared to HBBD and SML. UDP-D-glucose is converted to UDP-D-glucuronate which is a sugar nucleotide that is a precursor for several bacterial virulence factors.[20] UDP-N-acetylglucosamine also showed increased isotope incorporation in the BBD samples as compared to HBBD and SML. UDP-N-acetylglucosamine is used for peptidoglycan synthesis. Peptidoglycan forms the cell membrane in bacteria and is thicker in gram positive bacteria as compared to gram negative bacteria. BBD consists of a bacterial consortium with a mix of both gram positive and gram negative bacteria.[21] The increase in use of UDP-N-acetylglucosamine for BBD—as compared to the other samples—suggests that the bacterial consortium consists of more gram positive bacteria or much higher concentrations of cells because gram negative bacteria still use some peptidoglycogen.

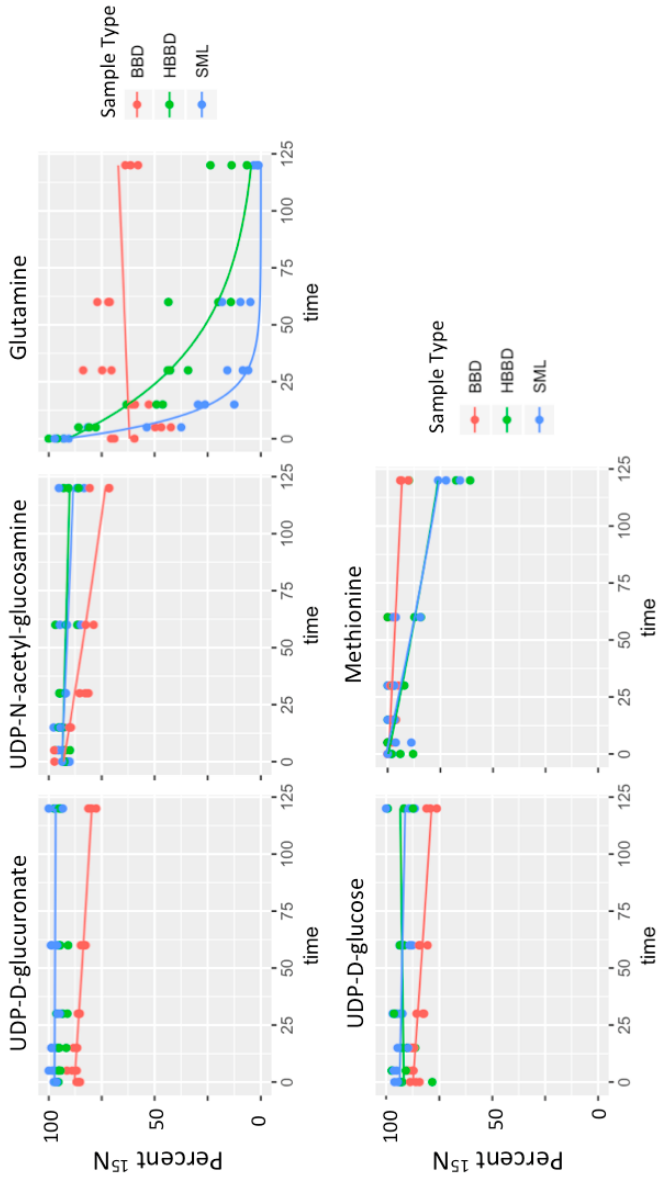

Figure 2.4: Flux for Ammonium Experiment

Table 2.1: Ammonium Flux Fits

Compound	Type	k	k (std dev)	A	A (std dev)
glutamine	BBD	-7.05E-04	9.75E-04	6.18E-01	3.56E-02
glutamine	HBBD	2.50E-02	3.42E-03	9.11E-01	4.56E-02
glutamine	SML	8.94E-02	1.35E-02	9.24E-01	5.63E-02
glutathione	BBD	1.27E-03	7.15E-04	9.66E-01	3.59E-02
glutathione	HBBD	-3.63E-04	2.78E-03	6.17E-01	9.93E-02
glutathione	SML	-1.69E-03	2.41E-03	7.22E-01	9.72E-02
glutathione disulfide	BBD	4.96E-04	7.17E-05	7.92E-01	3.11E-03
glutathione disulfide	HBBD	3.41E-04	3.63E-04	8.57E-01	1.72E-02
glutathione disulfide	SML	4.76E-04	4.46E-04	8.56E-01	2.09E-02
methionine	BBD	5.47E-04	1.03E-04	9.95E-01	5.58E-03
methionine	HBBD	2.25E-03	5.23E-04	9.97E-01	2.54E-02
methionine	SML	2.39E-03	3.84E-04	1.01E+00	1.86E-02
UDP-D-glucose	BBD	8.79E-04	1.39E-04	8.74E-01	6.46E-03
UDP-D-glucose	HBBD	-1.50E-04	3.00E-04	9.17E-01	1.57E-02
UDP-D-glucose	SML	2.32E-04	2.25E-04	9.36E-01	1.17E-02
UDP-N-acetyl-glucosamine	BBD	2.00E-03	2.63E-04	9.31E-01	1.21E-02
UDP-N-acetyl-glucosamine	HBBD	3.12E-04	1.77E-04	9.36E-01	9.17E-03
UDP-N-acetyl-glucosamine	SML	4.98E-04	2.21E-04	9.39E-01	1.14E-02
UDP-D-glucuronate	BBD	8.12E-04	1.14E-04	8.78E-01	5.37E-03
UDP-D-glucuronate	HBBD	NA	NA	NA	NA
UDP-D-glucuronate	SML	4.73E-05	1.18E-04	9.73E-01	6.48E-03

Methionine was another metabolite from the ammonium experiment that showed a decrease in isotope labeling in BBD as compared to HBBD and SML. Methionine is involved in sulfur metabolism, being one of two amino acids containing sulfur. It is a precursor to protein synthesis and a product of sulfur assimilation.

As BBD develops the amount of sulfate reducing bacteria increases and diversifies.[22] Richardson et al. found that the BBD communities of bacteria were much more diverse than in SML samples.[2] There may be regional differences in the BBD consortium based on the diversity even between two black band diseased colonies of the same species on the same reef. Once the disease has progressed from a cyanobacteria patch (CP) to BBD, the disease becomes much more virulent and the migration of the mat increases. Differences in available nutrients and microbial consortia occur at different depths of the mat. The mat closest to the coral has anoxia, high sulfide concentration, and low pH. The BBD mat has an upper phototrophic zone and lower anoxic-sulfide high zone which is essential for virulence.[23-24] The ammonium experiment generated differences in flux rates for several metabolites when comparing healthy and diseased coral showing increased metabolic activity as the disease progresses.

Glucose Flux

Selected flux graphs for the glucose experiment are shown in figure 2.5 with the corresponding fits in table 2.2. The glucose experiment showed an increase in isotope incorporation for glyceraldehyde-3-phosphate in BBD. Glyceraldehyde-3-phosphate is produced during glycolysis. It is a precursor to glycerol and phospholipid synthesis. Flavin adenine dinucleotide (FAD) also showed an increase in isotope incorporation in BBD as compared to HBBD and SML. FAD acts as an electron carrier. FAD accepts two electrons from succinate to be converted into fumarate and gets reduced to $FADH_2$. Succinate and fumarate are intermediates in the TCA cycle. Similar to the ammonium flux experiment, the glucose flux experiment showed an increase in the flux rate for UDP-D-glucuronate in BBD as compared to HBBD and SML. The metabolic flux rate was higher in the glucose experiment than in the ammonium experiment. Glutamate

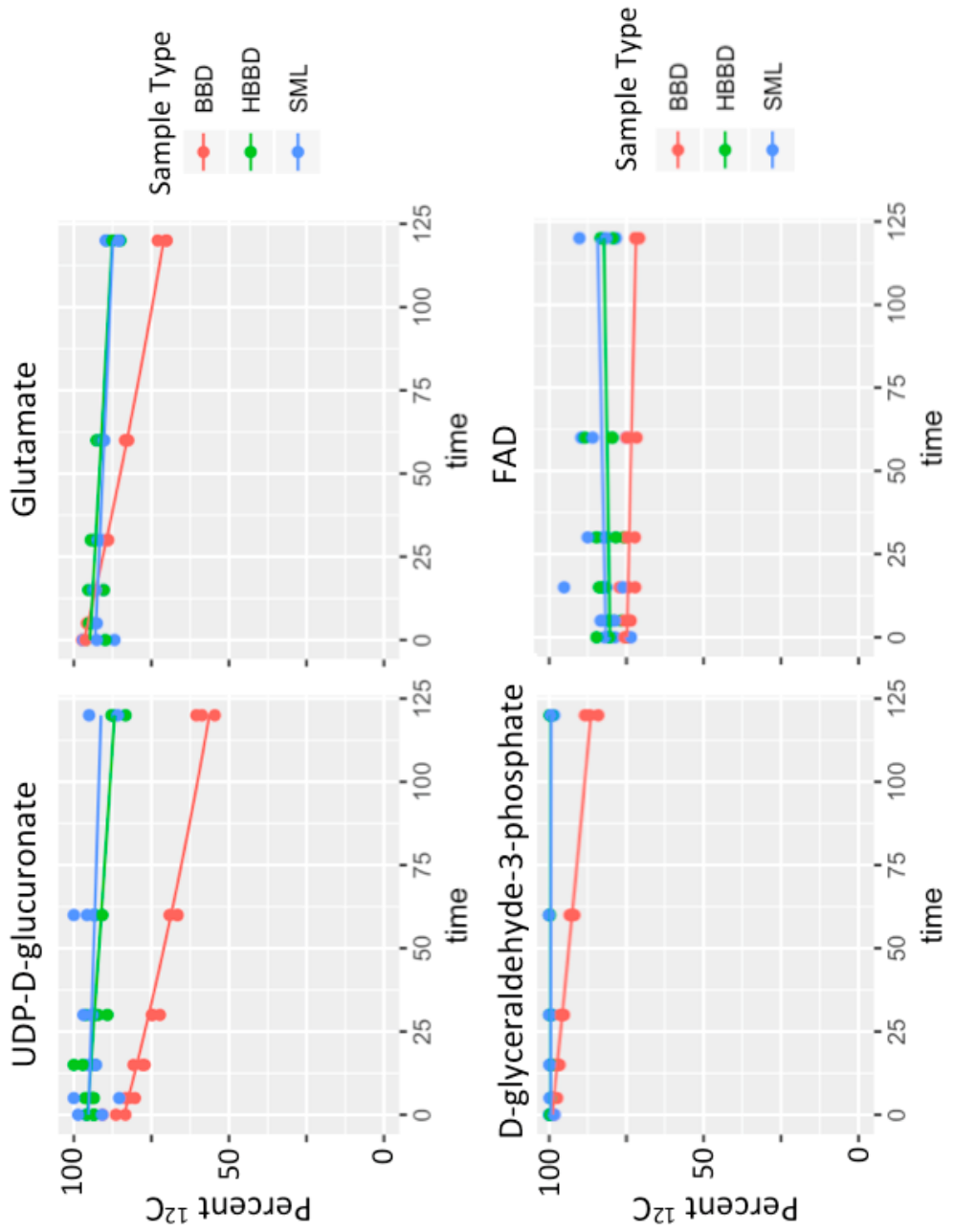

Figure 2.5: Flux for Glucose Experiment

Table 2.2: Glucose Flux Fits

Compound	Type	k	k (std dev)	A	A (std dev)
D-glyceraldehdye-3-phosphate	BBD	1.13E-03	6.10E-05	9.89E-01	3.17E-03
D-glyceraldehdye-3-phosphate	HBBD	1.37E-06	1.59E-05	9.96E-01	8.93E-04
D-glyceraldehdye-3-phosphate	SML	1.25E-05	3.68E-05	9.95E-01	2.06E-03
FAD	BBD	3.54E-04	1.14E-04	7.49E-01	4.69E-03
FAD	HBBD	-2.03E-04	2.42E-04	8.03E-01	1.11E-02
FAD	SML	-2.70E-04	3.86E-04	8.16E-01	1.81E-02
glutamate	BBD	2.57E-03	4.96E-05	9.67E-01	2.29E-03
glutamate	HBBD	6.51E-04	1.25E-04	9.48E-01	6.40E-03
glutamate	SML	5.25E-04	1.38E-04	9.31E-01	7.03E-03
UDP-D-glucuronate	BBD	3.31E-03	2.47E-04	8.38E-01	9.38E-03
UDP-D-glucuronate	HBBD	7.99E-04	1.61E-04	9.56E-01	8.27E-03
UDP-D-glucuronate	SML	3.60E-04	2.53E-04	9.53E-01	1.33E-02

also has a higher flux rate as compared to HBBD and SML; however, the rate was lower than the ammonium experiment.

Acetate Flux

Selected flux graphs for the acetate experiment are shown in figure 2.6 with the corresponding fits in table 2.3. The last isotope flux experiment used labeled acetate. Malate, aspartate, and succinate showed higher rates of isotope incorporation in BBD as compared to healthy coral. Acetate is a precursor to malate, succinate, and aspartate via the TCA cycle. Acetate enters the TCA cycle then downstream in the TCA cycle succinate in converted to fumarate which is converted to malate which is converted to oxaloacetate and then to aspartate. Aspartate is a precursor for methionine. Acetate is also used in the conversion of succinyl-coa to succinate. The flux results from the isotopic labeling of acetate show its incorporation through the TCA cycle. Glutathione disulfide (GSSG) also showed more isotope incorporation in BBD than healthy coral in the acetate experiment. The GSH/GSSG ratio is used as an indicator of oxidative stress.[25] Di-hydroxy-acetone phosphate (DHAP) also had increased isotope incorporation in BBD as compared to healthy coral. DHAP is involved in glycolysis and is converted to glycerol-3-phosphate from fructose-1, 6-bisphosphate. DHAP is a precursor to pyruvate and the TCA cycle.

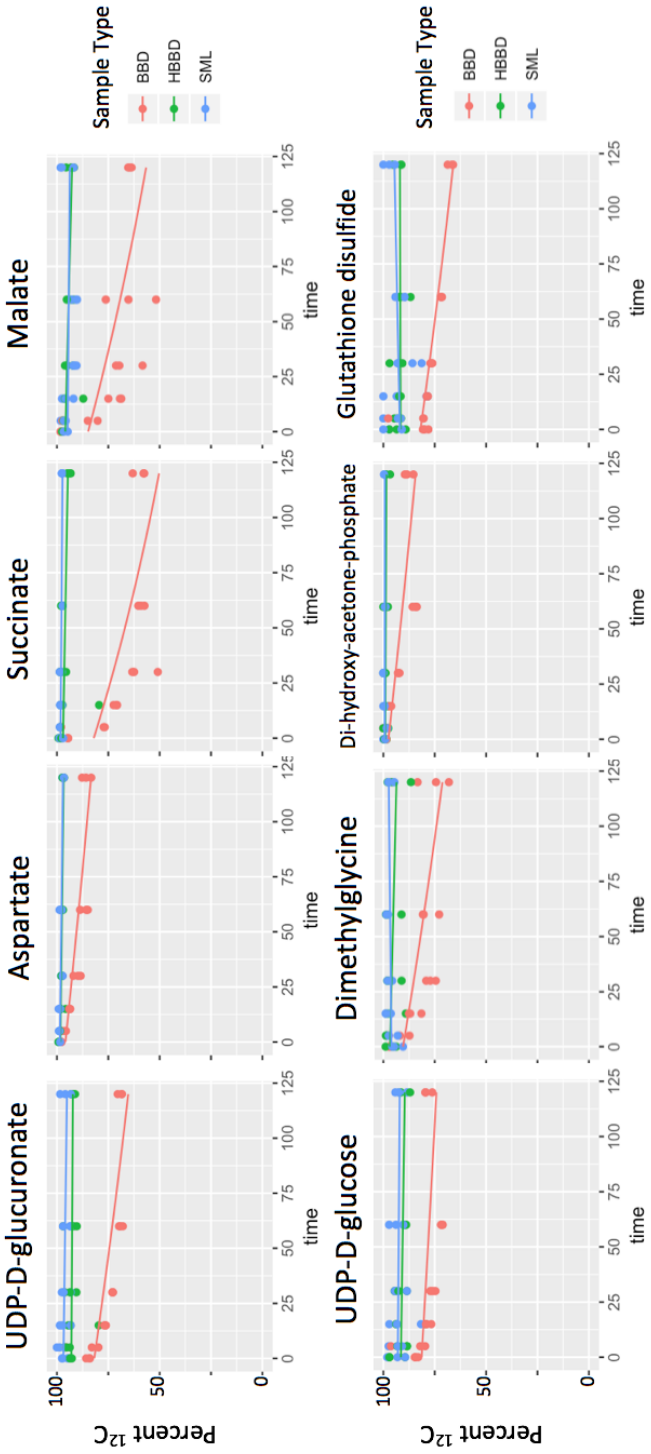

Figure 2.6: Flux for Acetate Experiment

Table 2.3: Acetate Flux Fits

Compound	Type	k	K (std dev)	A	A (std dev)
aspartate	BBD	1.16E-03	1.70E-04	9.59E-01	8.52E-03
aspartate	HBBD	1.32E-04	4.13E-05	9.83E-01	2.27E-03
aspartate	SML	1.33E-04	3.17E-05	9.86E-01	1.75E-03
dihydroxy-acetone-phosphate	BBD	1.21E-03	2.19E-04	9.75E-01	1.11E-02
dihydroxy-acetone-phosphate	HBBD	4.68E-05	5.51E-05	9.91E-01	3.07E-03
dihydroxy-acetone-phosphate	SML	4.95E-06	3.65E-05	9.93E-01	2.05E-03
dimethylglycine	BBD	1.98E-03	4.80E-04	9.04E-01	2.15E-02
dimethylglycine	HBBD	2.76E-04	2.24E-04	9.67E-01	1.20E-02
dimethylglycine	SML	-1.14E-04	1.30E-04	9.61E-01	7.13E-03
glutathione disulfide	BBD	1.81E-03	3.58E-04	8.19E-01	1.47E-02
glutathione disulfide	HBBD	-3.21E-05	2.64E-04	9.16E-01	1.37E-02
glutathione disulfide	SML	-2.36E-04	3.80E-04	9.20E-01	2.01E-02
malate	BBD	3.38E-03	9.93E-04	8.48E-01	3.80E-02
malate	HBBD	2.88E-04	1.59E-04	9.59E-01	8.43E-03
malate	SML	9.32E-05	1.81E-04	9.48E-01	9.64E-03
succinate	BBD	4.08E-03	1.07E-03	8.21E-01	3.77E-02
succinate	HBBD	2.04E-04	2.72E-04	9.70E-01	1.47E-02
succinate	SML	8.25E-05	3.04E-05	9.82E-01	1.68E-03
UDP-D-glucose	BBD	7.74E-04	4.13E-04	8.14E-01	1.80E-02
UDP-D-glucose	HBBD	1.73E-04	2.61E-04	9.15E-01	1.33E-02
UDP-D-glucose	SML	7.99E-05	2.54E-04	9.29E-01	1.33E-02
UDP-D-glucuronate	BBD	1.88E-03	4.18E-04	8.18E-01	1.70E-02
UDP-D-glucuronate	HBBD	6.59E-05	2.37E-04	9.30E-01	1.24E-02
UDP-D-glucuronate	SML	1.49E-04	1.28E-04	9.68E-01	6.96E-03

Conclusions

Previous studies have shown that the bacteria in the black band are a complex consortium that differs from the bacteria in healthy or dead parts of the coral reef.[3,5] The environmental conditions of the coral reef such as pH, temperature, and available sunlight can increase the virulence of the black band bacteria. The flux experiments revealed a faster metabolic rate for diseased coral samples when compared to healthy coral. Knowing what metabolites are most used by the bacterial consortium and what pathways they are involved in may be useful in further understanding coral disease. Coral reefs are an important oceanic habitat to many species and they are quickly becoming extinct. Human effects on the ocean could be contributing to the decline in coral reefs as well as rising sea temperatures.[1] Coming up with a way to combat coral diseases will be as complex as the consortium itself.

The R package developed for this project can be applied to other flux experiments to shorten data analysis time and help researchers analyze data. In the future, more functions can be added to this package to incorporate pathway mapping for key metabolites.

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Appendix

FluxR Script

```
library(devtools)
install_github("adsteen/metablize", ref="dev")
library(metabalize)

library(ggplot2)
library(reshape2)
library(plyr)
library(knitr)

# Load package by hand
source("R/calc_12C.R")
source("R/calc_ks_and_plot.R")
source("R/exp_coef.R")
source("R/fit_raw.R")
source("R/generate_exp_guess.R")
source("R/generate_facet_formula.R")
source("R/get_preds.R")
source("R/key_fn.R")
source("R/metabalize.R")
source("R/plot_single_metabolite.R")
source("R/plot_timecourse.R")
source("R/read_long.R")
source("R/read_MAVEN.R")
source("R/safe_NLS_pred.R")
source("R/safe_NLS.R")

#######
# DATA SETUP
#######

# Set data and key filenames
data.fn <- "data.csv"
key.fn <- "data_samplekey.csv"

# Read the raw data
raw_list_glucose <- read_MAVEN(data.fn=data.fn, key.fn=key.fn)

# Set the replicates to a factor
raw_glucose <- raw_list_glucose$raw_data
raw_glucose$replicate <- as.factor(raw_glucose$replicate)

# Pull out the 12C or 14N peaks (lighter isotope)
# Note that calc_12C identifies 12C OR 14N peaks (lowest mass isotope)
```

```
raw_glucose <- calc_12C(raw_glucose)


# Filter 12C or 14N peaks from 13C or 15N-containing peaks
C12_only <- subset(raw_glucose, is.12C==TRUE)

##########
# CREATE NLS FITS AND PREDICTIONS
##########

# Make nls fits for each compound
nls_fits_12C <- dlply(C12_only,
                c("compound", "treatment", "sample.type"), safe_NLS)


#### Make 'predicted' fits through each data set (i.e., the fit line)

# Define the 'domain' for predicted fits
dom <- seq(from=0, to=max(C12_only$time), length.out = 100)
# Make the predicted fits
pred_fits_12C <- ldply(nls_fits_12C, safe_NLS_pred, domain=dom)


# Find the number of cases where the computer couldn't fit
bad_fits <- is.na(nls_fits_12C)
frac_bad_fits <- sum(bad_fits)/length(nls_fits_12C)
print(frac_bad_fits)

#############
# DATA OUTPUT: Plotting and writing table of values
#############

# Make one, giant graph of the natural isotope-vs-time data
p_raw_huge <- ggplot(C12_only, aes(x=time, y=relative.ion.count, colour=replicate, shape=treatment)) +
 geom_point() +
 geom_line() +
 facet_grid(treatment~compound, scales="free_y") +
 theme(text=element_text(size=6))
print(p_raw_huge)
ggsave("plot.png", width=50, height=3, units="in", dpi=150,limitsize=FALSE)

# Make individual plots of each metabolite
all_plot_list <- dlply(C12_only, c("compound", "treatment"), plot_single_metabolite,
                print.plots=FALSE, save.plots=TRUE, base.fn=("plots/data"),
                predictions=pred_fits_12C,
                height=2.5, width=4, units="in", dpi=150)

# Get NLS fit parameters
```

```
coefs_df <- ldply(nls_fits_12C, exp_coef)
names(coefs_df)[4:8] <- c("A.est", "k.est", "A.std.err", "k.std.err", "n")
# Drop the ones where it couldn't calculate a fit
coefs_df_good <- na.omit(coefs_df)

# Make a .csv file of the fit results
write.csv(coefs_df_good, "data/fits.csv", row.names = FALSE)
```

CHAPTER 3
CHEMICAL DIVERSITY OF LUCIBUFAGINS IN FIREFLIES

This chapter contains material expected to be part of a publication by:
Marion, Z..; Farmer, A.T.; Campagna, S.R.

ZM designed the experiment and collected the samples. ATF developed the UPLC/PDA/MS method, analyzed the data, and wrote the following. ZM wrote the R scripts.

Abstract

Lucibufagins (LBGs) are a class of cardiotonic steroids found in fireflies (*Lampyridae*) similar to bufadienolides found in several toad species. Fireflies use them as a chemical defense against predation. LBGs have a conserved steroid backbone but studies have shown a large variety of potential LBGs that fireflies can use for defense. Most species of fireflies synthesize their LBGs however the species *Photuris* cannot synthesize them and must sequester them from other species of fireflies. Almost 700 firefly samples were analyzed for both known and unknown LBG content and analyzed for potentially new LBGs and statistically investigated what conditions may be affecting the LBG composition.

Introduction

Method Development

Atmospheric Pressure Chemical Ionization

For molecules to be detected by the mass spectrometer, they must first be ionized and in the gas phase. The two most common types of ionization sources that can be easy coupled to mass spectrometers include electrospray ionization (ESI) sources and atmospheric pressure chemical ionization (APCI) sources. APCI can be used to analyze non-polar, thermally stable compounds. The eluent is sprayed through the APCI nozzle which is heated to assist in drying the droplets. N_2 gas is used to further dry the

droplets. A corona discharge needle is situated perpendicular to the spray and creates an ionization region that ionizes the droplets as they dry.[1,2]

APCI was used for this study because it's a better ionization source for nonpolar steroidal compounds that don't ionize as well using ESI.[3] Like ESI, APCI can be easily coupled to a liquid chromatography (LC) system, which can separate compounds based on molecular properties prior to analysis by the mass spectrometer. This reduces the total amount of ions being introduced at a time, which increases sensitivity.

Photodiode Array Detection

Photodiode array detectors (PDAs) detect absorbance that occurs as a result of electron transitions. The flow cell that the analyte travels through is irradiated by a specific wavelength or a wavelength range that is then diffracted by a grating to an array of photodiodes for detection.[4] PDAs are non-destructive and can be used in-line with mass spectrometry and liquid chromatography. Since LBGs absorb at a wavelength of 300nm a PDA was used in this study to couple with mass spectrometry for potential novel LBG identification.[5] The LC eluent passes through the PDA and then to the APCI source. This yields several layers of information: the retention time from liquid chromatography, the UV-vis spectra from the PDA, and the *m/z* ratios from the mass spectrometer.

Bufadienolides in Fireflies

Fireflies (*Lampyridae*) are a type of beetle well known for their mating light displays. Though beautiful, these light displays increase their visibility to predators. Hemolymph is the invertebrate equivalent of mammalian blood and is secreted by fireflies on their exoskeleton when they are harassed or threatened.[6] Their hemolymph contains defensive molecules: a betaine, N-methylquinolinium 2-carboxylate, and compounds coined as lucibufagins (LBGs).[7] LBGs are cardiotonic steroids like the bufadienolides found in some plants and animals but referred to as LBGs when in fireflies. Their basic steroid backbone can have modifications leading to a variety of LBGs found in fireflies. The LBG composition can vary greatly between firefly species and location. Presumably

fireflies synthesize these LBGs from their environment, however, the exact origin and mechanism of LBG synthesis is unknown.[8] Most likely these LBGs are synthesized from substrates they ingest.

When fireflies release hemolymph it coagulates quickly. Once it coagulates it impedes predators from eating them because their mouths are hampered by the sticky excretions. These excretions also contain the distasteful and poisonous LBGs.[9] Depending on the firefly, ingesting just one could be lethal to a small pet.[10] Research by Blum et al. shows that hemolymph clots rapidly meaning the hemolymph itself, even without LBGs is a deterrent to predation.[11]

Their obvious lighted courtship makes fireflies easy targets for predators, which can be other invertebrates, small mammals, or even a different species of firefly. *Photuris* is a firefly species that has been nicknamed the “femme fatale” firefly because of its propensity to eat other fireflies. *Photuris* prey on other fireflies for LBGs. Eisner et al. showed that *Photuris* fireflies do not contain LBGs until they have eaten other fireflies.[5] They cannot synthesize LBGs therefore they sequester them from other species that can. They attack *Photinus* males by mimicking *photinus* female flash patterns, aerial style (aka hawking), or steal them from spider’s webs.[12]

The purpose of this study was a) to qualitatively measure the amount and the presence/absence of known LBGs in different firefly species from different geographic locations and b) to measure any novel LBGs that haven’t been reported before.

First an UPLC-APCI-MS method was developed on the Orbitrap Exactive Plus. The method was developed using bufadienolide standards and the data was processed based on known masses. Figure 3.1 shows two of the bufadienolides used for method development. Then the interest turned to investigating unknown LBG compounds. The original data set consisted of almost 700 different firefly samples. A subset of 50 samples was chosen based on its diversity of compounds and was run on the Orbitrap

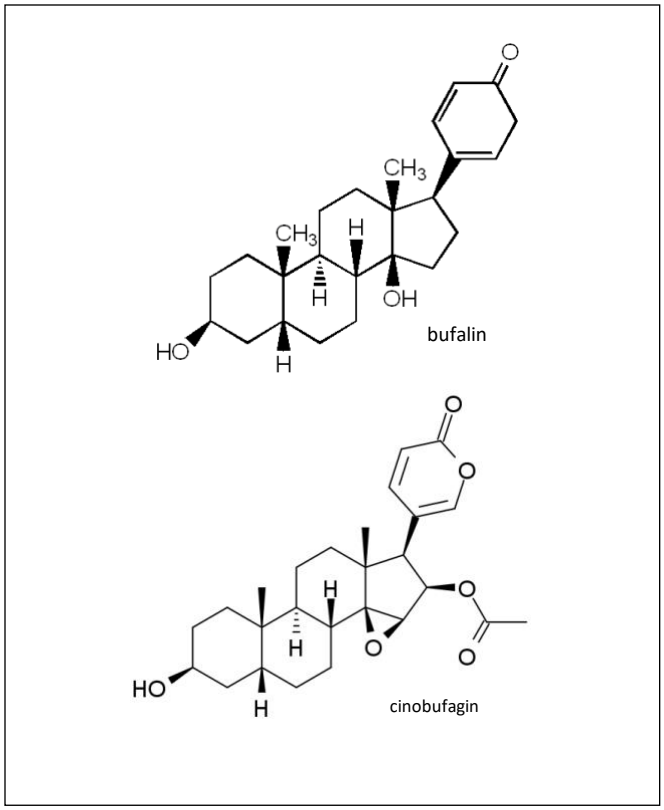

Figure 3.1: Selected bufadienolides structures

again, this time with a photodiode array detector inline. LBGs are UV active with a signature peak at approximately 300 nm.[5] This way, when the mass spectrometry data was processed the unknown masses could be narrowed down using the retention times from the PDA when UV active compounds were present. The original mass list of unknown compounds detected during the full scan analysis was extensive including over 40,000 spectral features. Of those, 20,480 unknown masses matched the retention times of UV active compounds and were selected for computational processing to remove isotope and adduct. The remaining 653 masses were then further pruned by selecting only those with a mass large enough to correspond to an LBG structure. The final mass list used for reprocessing the original data set contained 174 masses down from 20,480 selected masses.

Materials and Methods

Sample Collection and LBG extraction

Lampyrid communities were sampled from multiple locations in North America and one location in Belgium. Species, sex, county, state, collection date, dry weight, and nocturnal/diurnal information for all samples used for statistical analysis is shown in Table 3.8. Individuals were dried under vacuum and weighed to the nearest microgram prior to extraction. The samples were defatted with hexane. Then, individual samples were sequentially extracted by sonication with methanol and then 20% acetonitrile (ACN). The solvents were removed via vacuum centrifugation and the extracts were re-suspended with 10 mM of an internal standard (6-methylcoumarin) in 1 mL of 2% acetonitrile and 0.1% formic acid. After being re-suspended the sample was sonicated for 5 minutes, centrifuged at 13.2 rpm for 5 minutes, then 10 µL of the supernatant was injected for analysis. Following extraction, each sample was analyzed for the presence of bufadienolides/LBGs using UPLC-APCI-MS.

UPLC/PDA/MS methods

LBG extracts were separated on a Kinetex C18 column (30 mm x 2.1 mm, 2.6 µm) (Phenomenex, Torrance, CA, USA) connected to an Ultimate 3000 autosampler and UHPLC pump and an Exactive Plus benchtop Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an atmospheric pressure chemical ionization (APCI) probe. Separations ran for 16 min with mobile phase A and B consisting of 0.1% formic acid in water (H_2O) and ACN respectively. The gradient started at 98% B for 1 min; from 1 to 5 min decreased to 75% B, from 5 to 12 min decreased to 38.6% B, from 12.001 min to 16 min maintained at 98% B. The column oven temperature was maintained at 30°C and the temperature of the autosampler was set to 4°C. The same LC conditions and buffers were used for all MS experiments with a flow rate of 0.290 mL/min. The external standards used for method development were bufalin, bufotalin, resibufogenin, cinobufagin, and cinobufotalin.

The spray voltage was set to 5 kV, the heated capillary was set at 175 °C, and the probe heater was set at 350 °C. The sheath gas flow was set to 60 units and the auxiliary gas set to 15 units. These conditions were held constant for all acquisitions. External mass calibration was performed using the standard calibration mixture and protocol from ThermoFisher approximately every 2 days. The mass spec analysis was operated at a resolution of 140,000 with a scan range of 100-700 m/z. Each sample was run in positive mode.

A subset of samples with the most LBG diversity was run in line with a PDA detector with a range from 220-400 nm, a sampling rate of 5, and spectral resolution of 1.2 nm. The data was processed for 80 known bufadienolides/LBGs (and their isomers) to within 5 ppm. An external calibration curve of 5 standards was used to estimate concentrations. 6-methyl-coumarin was used as an internal standard to align peaks between the mass spectrometer and the photodiode array detector and as a quality control standard for mass spec injections.

Data Analysis

A total of 691 samples were run on MS. A subset of 50 samples showing the most compound diversity was run on PDA in line with the mass spectrometer.

MAVEN was used to process data for known LBGs.[13] The R package XCMS was used to process the unknown mass spectrometry data.[14] The R package CAMERA was used to remove isotopes and adducts from the unknown masses.[15] Metaboanalyst was used to generate Partial Least Squares discriminant analysis (PLSDA) plots and variable importance in projection (VIP) scores.[16] R was used to generate the redundancy analysis (RDA) plot.[17]

Results/Discussion

All of the firefly samples had at least species, location, nocturnal/diurnal, and sex/egg information. Out of all the samples analyzed, some were excluded from post processing analysis. Larva were excluded because of their small sample size and any species that had less than 3 replicates was excluded; therefore, a total of 678 samples were used for comparisons. Partial least squares discriminant analysis (PLSDA) was chosen as a multivariate model to investigate the unknown data. There were 174 unidentified features to investigate for 678 samples. PLSDA was used because in this case there are more dependent variables than independent variables. PLSDA aims to show if the samples are different and which variables are contributing the most to that difference. Variable importance in projection (VIP) scores measure a variable's importance to the PLSDA model. All PLSDAs were generated using the unidentified LBG masses.

The PLSDA in figure 3.2 shows the separation between females, males, and eggs for all species. If adult fireflies must acquire substrates to make LBGs then eggs wouldn't have as many or as diverse of an LBG composition as adults. The females and males for all species overlap while the eggs are separate. The PLSDA in figure 3.3 shows the separation between sex for just the *Photuris* fireflies. *Photuris* fireflies cannot synthesize LBGs and must sequester them from other species. *Photuris* females have been shown

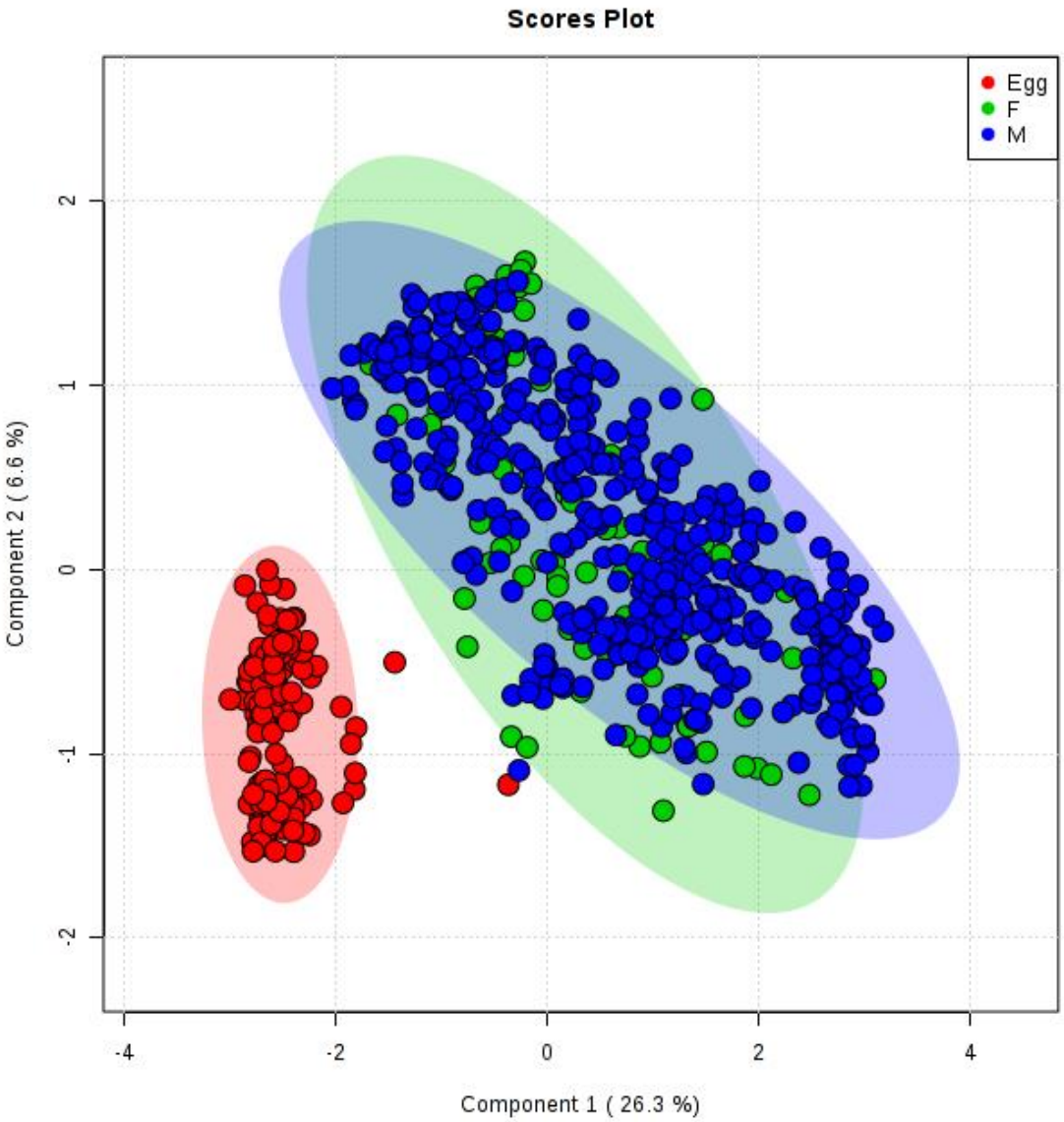

Figure 3.2: PLSDA of all species comparing females, males, and eggs

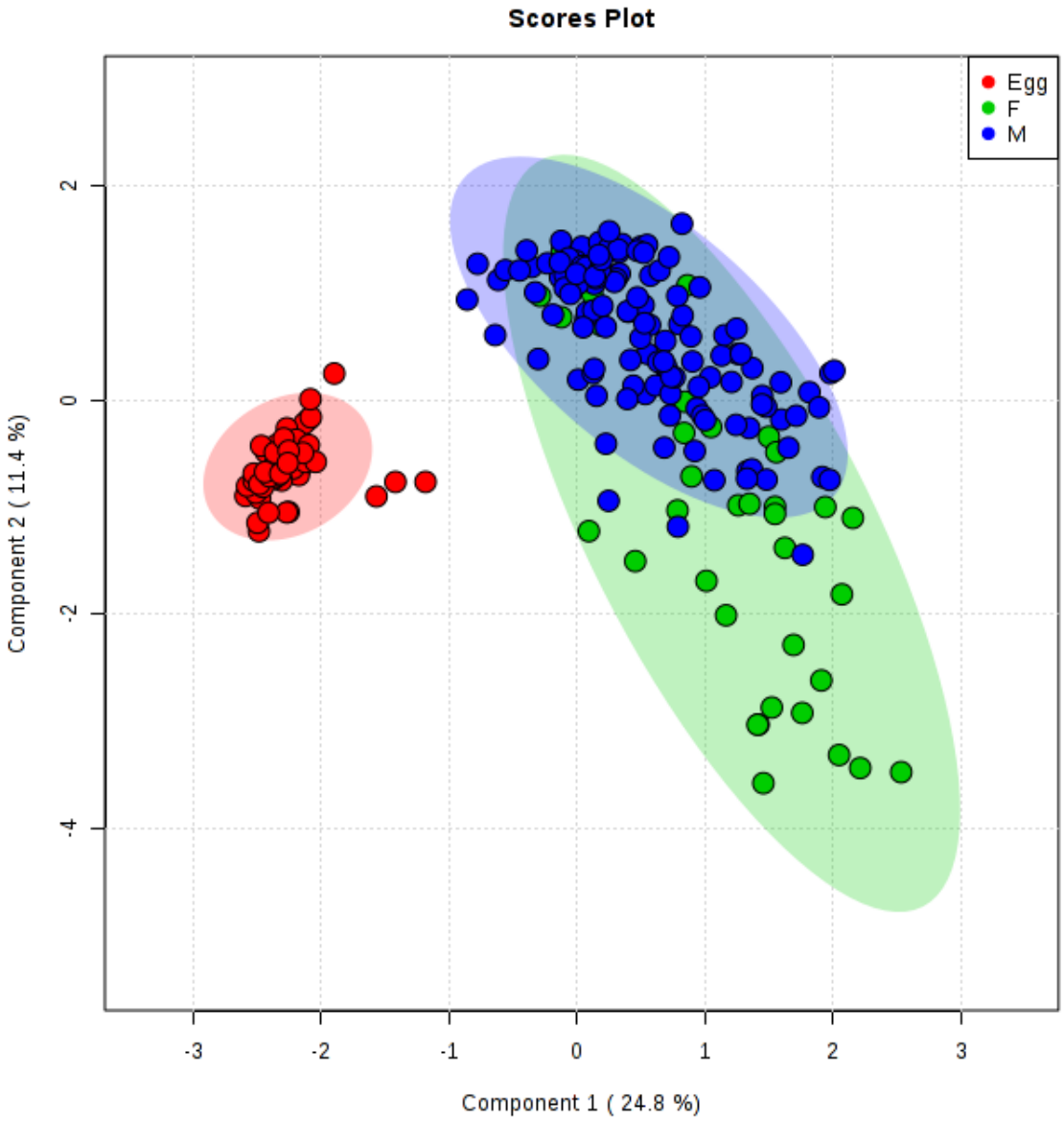

Figure 3.3: PLSDA of *Photuris* females, males, and eggs

to mimic flash patterns to lure males of other species and they have more LBGs than male *Photuris* who also must sequester LBGs.[5] A study by Gonzalez et al shows *photuris* pass acquired LBGs to their offspring.[18] *Photuris* fireflies can pass on their chemical defenses by coating LBGs onto their eggs to protect them from predators. The more chemical defenses the eggs have the less appetizing they are to potential predators. There is slightly more separation of the male and female fireflies for *photuris* species which correlates to these findings.

Figure 3.4 shows the PLSDA for all species separated by county. The counties where samples were collected were Anderson, Berks, Blount, Butler, Forest, Knox, and Wynegem. Berks, Butler, and Forest are located in Pennsylvania; Anderson, Blount, and Knox are located in Tennessee; and Wynegem is located in Belgium. Although the exact mechanism of LBG synthesis in fireflies isn't known; presumably fireflies synthesize their LBG from substrates they consume. Therefore, we would expect separation between locations based on what food is available in these locations. However, as seen in the RDA analysis in figure 3.5, the majority of differences is explained by both the location and the species. The RDA plot was made using the presence/absence data of all identified potential LBGs. Figure 3.6 is a PLSDA showing the separation of fireflies collected in Tennessee counties. It's interesting to note that even within a state there are differences based on location.

Photinus are the most common firefly and synthesize their own LBGs. Figure 3.7 shows the separation for just *photinus* species based on counties which shows more variation than when all the species are compared together. This could be due to the exclusion of *photuris* species which must acquire their LBGs, and diurnal species like *lucidota* which contain less LBGs overall. Studies have shown that the more LBGs a firefly contains then the more they are protected from predators and diurnal species need less protection. [5]

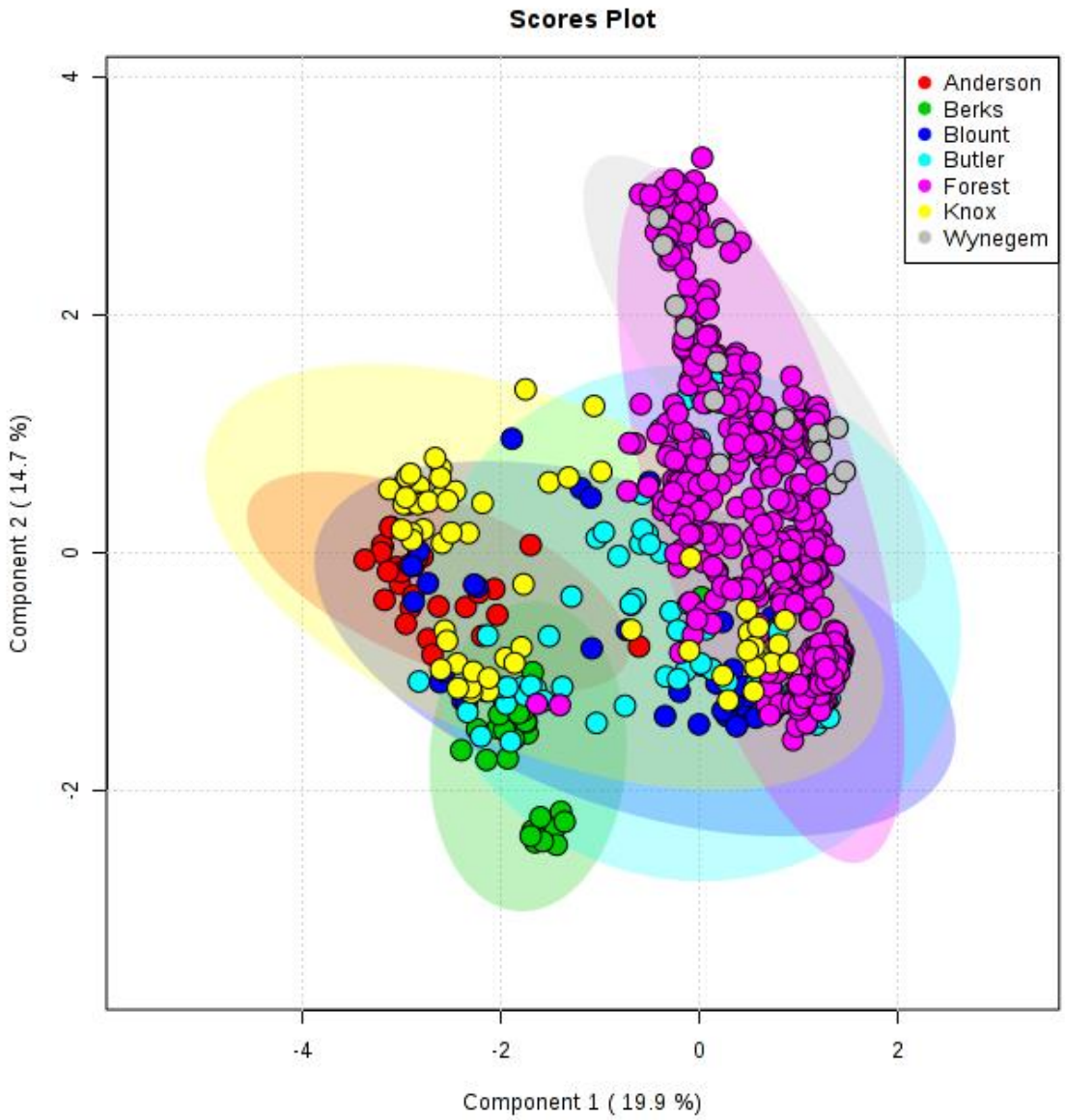

Figure 3.4: PLSDA comparing counties

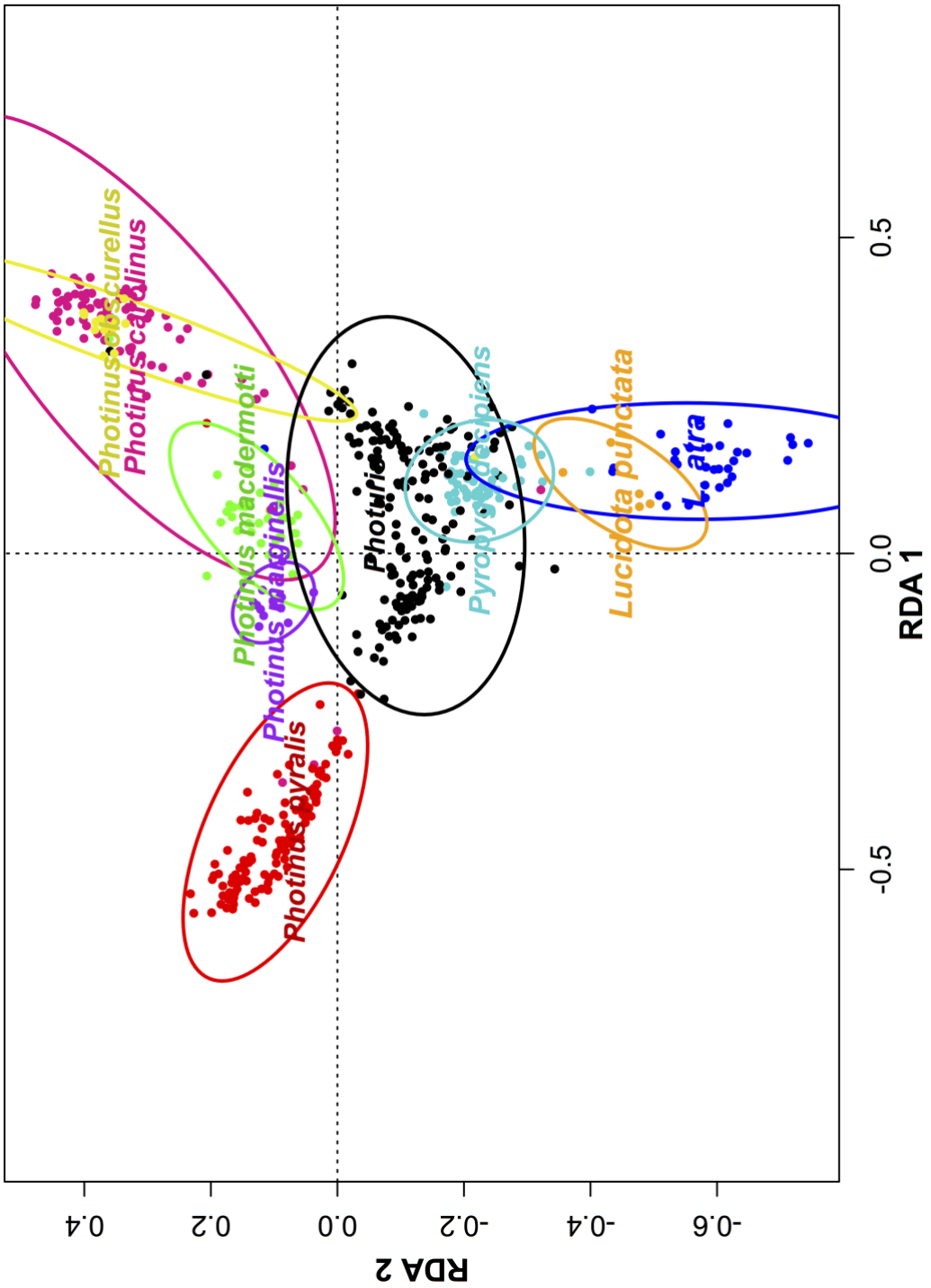

Figure 3.5: RDA analysis of presence/absence data constrained by species and location

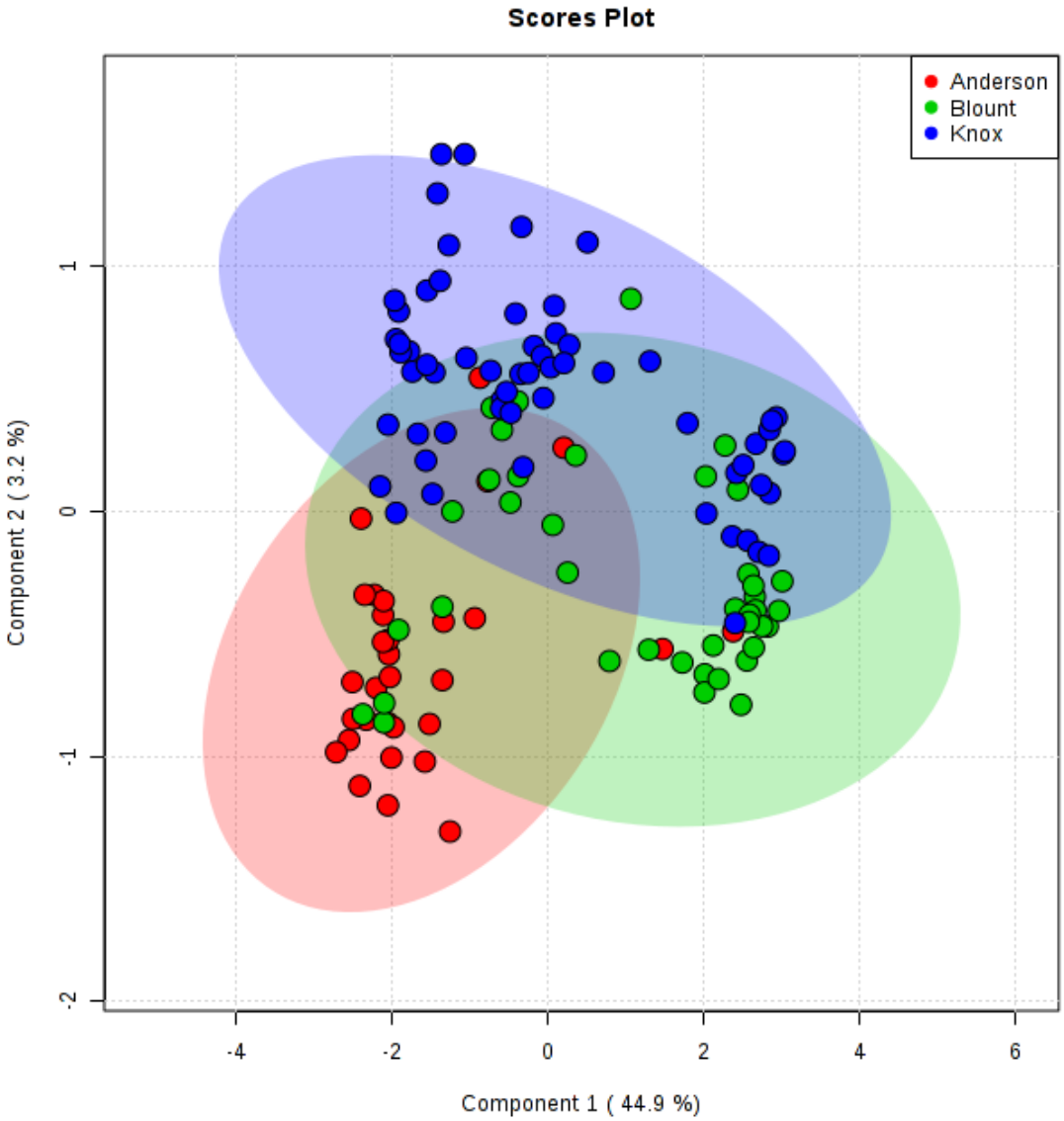

Figure 3.6: PLSDA of Tennessee counties

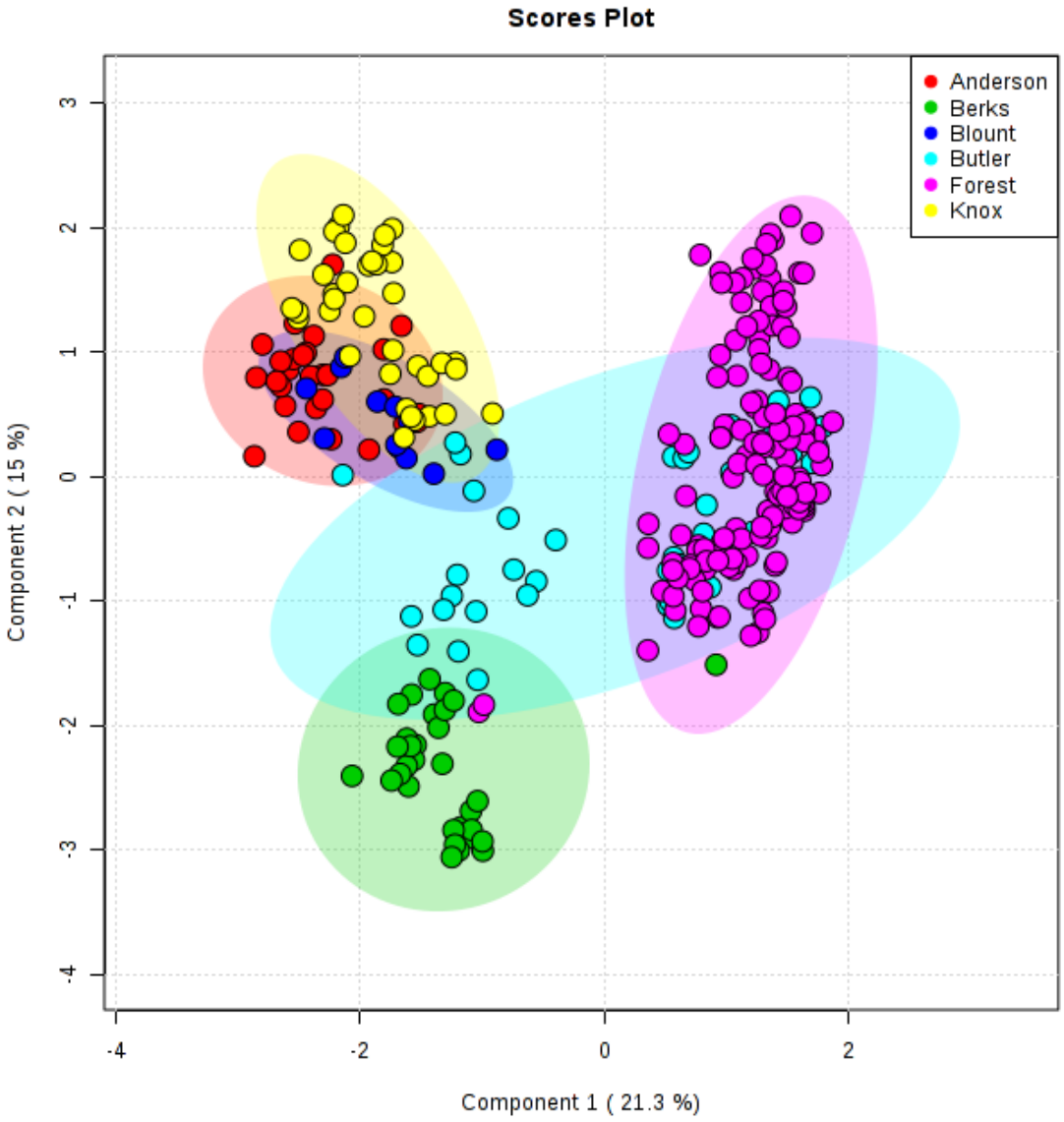

Figure 3.7: PLSDA of Photinus counties

Figure 3.8 shows the PLSDA comparing the nocturnal and diurnal species and there is some separation between the groups. The nocturnal species are more visible to predators so they need to have a larger amount of LBGs to protect them.[19] Diurnal fireflies use pheromones to attract mates instead of light displays. By not using light shows for mating diurnal fireflies are less visible to predators therefore have a lesser need for chemical defenses against predators. Diurnal fireflies need less protection and that's reflected in the overall amount of LBGs they contain in their hemolymph.

Nocturnal fireflies are bioluminescent and use species-specific flashing patterns to attract mates.[20] A study by Lewis et. al. suggests that some ancient firefly species didn't develop bioluminescence whereas some newer species have over time lost bioluminescence. Nocturnal species visible breeding displays increased their visibility to predators but may also serve as a visual reminder to predators to avoid them. Many predators can recognize undesirable prey by remembering visible cues. The flashing of fireflies may deter them knowing that they are sticky and distasteful when attacked and eaten. Research by Lewis et al. advises that firefly flashing may help predators know to avoid them because they associate the flashing with the distasteful LBGs.[21]

Research conducted by Gronquist et al. (2006) investigated *Lucidota atra*, a diurnal firefly species. Although *Lucidota atra* fireflies contain LBGs, they have less than their nocturnal relatives. They propose that *Lucidota atra* may have evolved to diurnality to avoid being eaten by the nocturnal *photuris* fireflies that prey on other firefly species.[19]

Evolutionary energy budget explains the cost benefit balance of species traits changing over time. Nocturnal fireflies that developed bioluminescence to communicate also developed larger eyes and smaller antennas than diurnal fireflies that communicate by chemical pheromones. Research by Stranger-Hall et. Al. supports that mate selection increases the main sensors used for breeding due to the balance of energy budget.[22-23] Though diurnal fireflies don't need as many LBGs for protection they still benefit from the LBGs they synthesize.

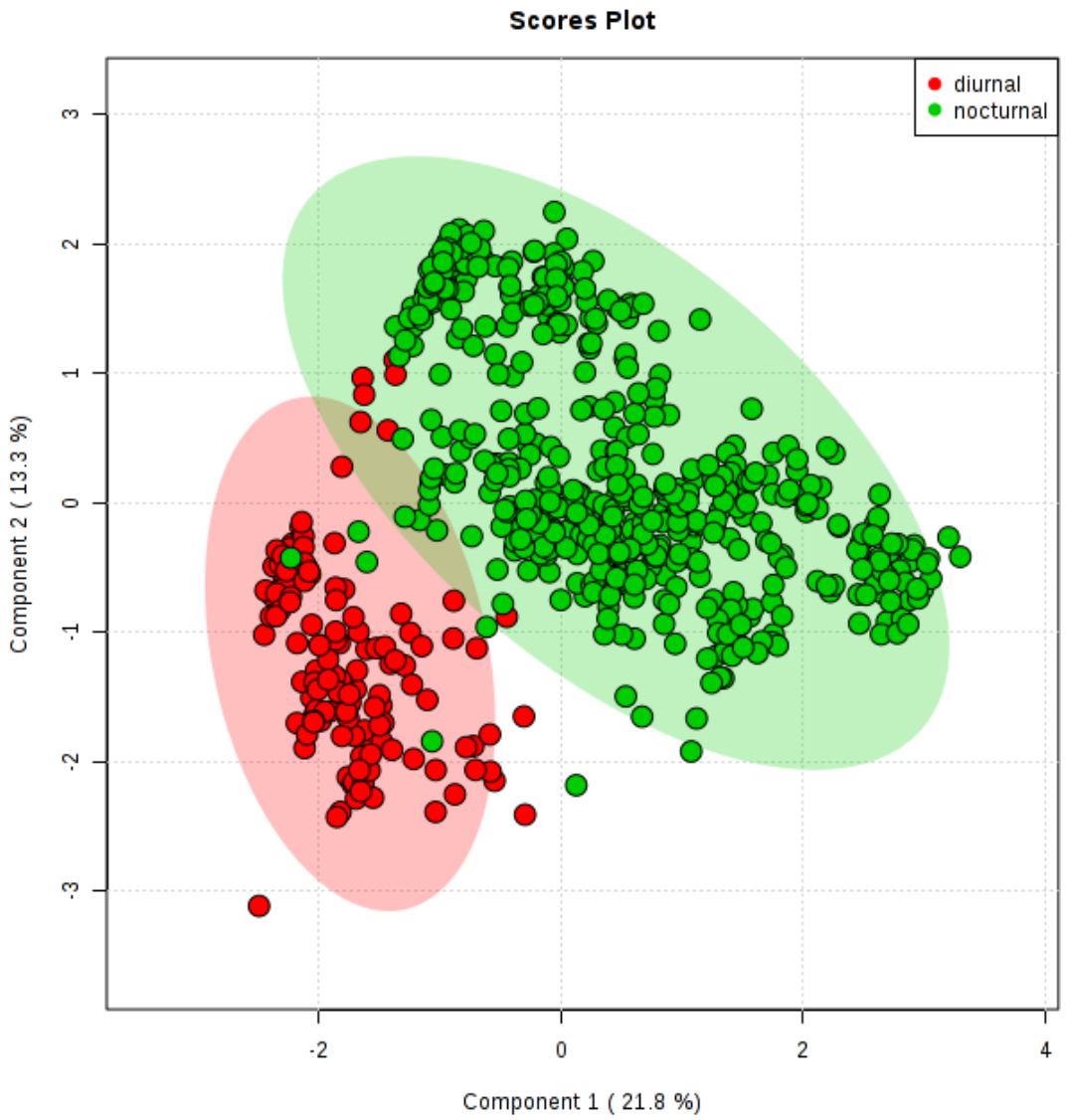

Figure 3.8: PLSDA of diurnal and nocturnal species

When comparing the total amount of unidentified LBGs as well as total presence of unidentified LBGs there are more present in nocturnal species than diurnal species. Based on recent literature and the separation seen here the difference in LBG amounts and diversity between species may be due to the difference in mating styles—light shows for the nocturnal species and pheromones for the diurnal species.

Tables 3.1 through 3.6 show the VIP scores corresponding to the PLSDA plots. Analysis of the top 10 VIP scores for the PLSDAs shown reveal there are 17 masses that are top contributors for more than one comparison. ChemCalc was used to calculate potential molecular formulas.[24] Molecular formulas were calculated using the elemental constraints $C_{(20\text{-}100)}$, $H_{(20\text{-}100)}$, $O_{(2\text{-}100)}$, and $N_{(0\text{-}3)}$ based on the bufadienolide and LBG structures that have been identified. Fourteen of the masses generated molecular formulas using these parameters with mass differences less than 10 ppm. Six of those formulas had PubChem matches which are shown in table 3.7.[25]

These formulas can be investigated in the future to confirm their identity as LBGs and probe how and why they contribute to the differences seen between firefly's LBG content.

Table 3.1: Top VIP scores for PLSDA comparing females, males, and eggs

m/z	Comp. 1	Comp. 2
406.135	2.791	2.667
369.027	2.135	1.857
424.842	2.051	1.839
365.164	1.986	1.841
430.199	1.950	1.950
422.231	1.919	1.591
441.245	1.902	1.562
458.224	1.894	1.567
392.162	1.842	1.597
422.231	1.842	1.520

Table 3.2: Top VIP scores for *Photuris* females, males, and eggs

m/z	Comp. 1	Comp. 2
401.184	3.711	3.154
406.135	2.439	1.986
369.027	2.402	1.939
424.842	2.298	1.849
401.133	2.283	1.851
390.159	2.265	1.872
387.214	2.193	1.930
473.215	2.158	2.055
365.164	2.077	1.670
507.256	2.050	1.659

Table 3.3: Top VIP scores for counties

m/z	Comp. 1	Comp. 2
514.219	3.554	2.849
522.304	3.350	2.740
565.290	3.288	2.639
472.208	3.200	2.573
471.227	3.164	2.510
486.221	3.156	2.539
523.278	2.093	1.695
519.257	2.080	1.650
466.257	2.075	1.688
532.229	2.068	1.643

Table 3.4: Top VIP scores for Tennessee counties

m/z	Comp. 1	Comp. 2
466.221	2.444	0.911
458.882	2.154	1.018
463.277	2.019	1.151
408.158	1.984	0.721
476.240	1.944	1.497
408.158	1.935	0.713
486.221	1.860	0.843
466.257	1.843	0.859
420.273	1.831	0.900
504.284	1.714	0.683

Table 3.5: Top VIP scores *Photinus* counties

m/z	Comp. 1	Comp. 2
522.304	3.216	2.699
565.290	3.107	2.573
514.219	3.104	2.573
486.221	3.040	2.512
472.208	2.685	2.219
523.278	2.344	1.975
507.257	2.270	1.876
471.227	2.257	1.867
430.198	2.246	1.947
507.256	2.154	1.780

Table 3.6: Top VIP scores for diurnal and nocturnal species

m/z	Comp. 1	Comp. 2
551.273	2.776	2.569
549.267	2.399	2.451
466.257	2.014	1.611
471.227	1.935	1.581
468.257	1.932	2.195
358.156	1.913	1.558
391.170	1.820	1.414
392.162	1.790	1.526
374.151	1.790	1.549
401.133	1.783	1.383

Table 3.7: Potential annotations for unknown masses

m/z	Potential molecular formula
365.164	$C_{22}H_{23}NO_4$
392.162	$C_{24}H_{24}O_5$
466.257	$C_{25}H_{38}O_8$
471.227	$C_{26}H_{33}NO_7$
472.208	$C_{26}H_{32}O_8$
565.290	$C_{29}H_{43}NO_{10}$

Conclusion

As demonstrated here, fireflies can be an effective model for chemical diversity analysis. Chemical ecology is the study of chemically mediated interactions between organisms.[26-27] Fireflies communicate chemically with potential mates, other firefly species, and possible predators. The method used for this study could be applied to other invertebrates or vertebrates that have unique chemotypes or secondary metabolites reflecting changes in their genome or environment. Many classes of compounds have unique UV-vis absorptions that can be used to screen unknown spectral features to exclude masses that don't correspond to that absorbance.

The PLSDA analysis show differences between the LBG composition of nocturnal and diurnal fireflies. The effects that nocturnal vs diurnal behavior in fireflies have on their LBG composition should be further investigated for potentially novel naturally occurring compounds.

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Appendix

Conditional Matching Script

```
### aligns PDA peaks with all of the MS peaks. Alignment is
where MS.RT (mass spec retention time) falls within PDA
retention time window.

datMS<-read.csv("")
 names(datMS)
 newMS <- datMS[, c("sample", "peakMz", "rt", "rtmin", "rtmax",
"quality", "peakArea", "peakAreaCorrected")]

colnames(newMS)[c(3:5)] <- c("MS.rt","MS.rtmin", "MS.rtmax")

datPDA <- read.csv("")
names(datPDA)
newPDA<- datPDA[, -4]

########Loop looking for MS peaks that start and end within the
following interval: 10% of the peak width before PDA peak start
and end 20% of peak width after PDA peak end

width <- newPDA$PDA.end.adj - newPDA$PDA.st.adj
newPDA <- cbind(newPDA, width)

samps <- unique(newPDA$SampleName)
ret <- c()
i = 45
for(i in 1:length(samps)){
      MS <- newMS[which(newMS$sample == samps[i]),]
      PDA <- newPDA[which(newPDA$SampleName == samps[i]),]
      for(j in 1:dim(MS)[1]){
            for(k in 1: dim(PDA)[1]){
                        if(MS$MS.rt[j] >= PDA$PDA.st.adj[k] &
MS$MS.rt[j] <= PDA$PDA.end.adj[k]){
                                    tmp<-cbind(MS[j, c(1:8)],
PDA[k, c(2:8)])
                                    ret<-rbind(ret,tmp)
                  }
            }
      }
}
```

Table 3.8: Firefly sample information

ID	Species	Sex	County	State	Date Coll.	Dry Wt.	Nocturnal/Diurnal
PA001	*Photinus marginellis*	M	Butler	PA	6.14.2012	3.0188	nocturnal
PA002	*Photinus marginellis*	M	Butler	PA	6.14.2012	3.3313	nocturnal
PA003	*Photinus marginellis*	M	Butler	PA	6.14.2012	3.1534	nocturnal
PA004	*Photinus pyralis*	M	Butler	PA	6.14.2012	16.7175	nocturnal
PA005	*Photuris sp.*	F	Butler	PA	6.14.2012	19.3536	diurnal
PA006	*Photuris sp.*	F	Butler	PA	6.14.2012	10.0574	diurnal
PA007	*Photuris sp.*	F	Butler	PA	6.14.2012	30.0834	diurnal
PA008	*Photuris sp.*	F	Butler	PA	6.14.2012	19.95	diurnal
PA009	*Photuris sp.*	M	Butler	PA	6.14.2012	16.104	diurnal
PA010	*Photuris sp.*	F	Butler	PA	6.14.2012	14.9999	diurnal
PA011	*Photuris sp.*	M	Butler	PA	6.14.2012	18.7019	diurnal
PA012	*Photinus pyralis*	M	Butler	PA	6.14.2012	10.0931	nocturnal
PA013	*Photuris sp.*	M	Butler	PA	6.14.2012	14.5328	diurnal
PA014	*Photuris sp.*	F	Butler	PA	6.14.2012	11.1738	diurnal
PA015	*Photinus pyralis*	M	Butler	PA	6.14.2012	12.4556	nocturnal
PA016	*Photinus pyralis*	M	Butler	PA	6.14.2012	12.1368	nocturnal
PA017	*Photuris sp.*	M	Butler	PA	6.14.2012	10.6343	diurnal
PA018	*Photuris sp.*	F	Butler	PA	6.14.2012	28.0885	diurnal
PA019	*Photuris sp.*	M	Butler	PA	6.14.2012	11.5201	diurnal
PA020	*Photuris sp.*	M	Butler	PA	6.14.2012	18.6373	diurnal
PA021	*Photuris sp.*	M	Butler	PA	6.14.2012	10.8548	diurnal
PA022	*Photuris sp.*	M	Butler	PA	6.14.2012	22.0238	diurnal
PA023	*Lucidota atra*	F	Butler	PA	6.15.2012	15.5889	diurnal
PA024	*Photinus pyralis*	M	Butler	PA	6.15.2012	9.3962	nocturnal
PA025	*Photinus carolinus*	M	Butler	PA	6.15.2012	7.2229	nocturnal
PA026	*Photinus carolinus*	M	Butler	PA	6.15.2012	7.0556	nocturnal
PA027	*Photinus carolinus*	M	Butler	PA	6.15.2012	8.7615	nocturnal
PA028	*Photinus carolinus*	M	Butler	PA	6.15.2012	6.8589	nocturnal
PA029	*Photinus carolinus*	M	Butler	PA	6.15.2012	6.9578	nocturnal
PA030	*Photinus carolinus*	M	Butler	PA	6.15.2012	7.6564	nocturnal

Table 3.8: Continued

ID	Species	Sex	County	State	Date Coll.	Dry Wt.	Nocturnal/Diurnal
PA031	*Photinus carolinus*	M	Butler	PA	6.15.2012	11.1937	nocturnal
PA032	*Photinus carolinus*	M	Butler	PA	6.15.2012	7.0835	nocturnal
PA033	*Photinus carolinus*	M	Butler	PA	6.15.2012	7.7523	nocturnal
PA034	*Photinus carolinus*	M	Butler	PA	6.15.2012	8.562	nocturnal
PA035	*Photinus carolinus*	M	Butler	PA	6.15.2012	6.745	nocturnal
PA037	*Photinus pyralis*	M	Butler	PA	6.15.2012	11.5082	nocturnal
PA038	*Photinus pyralis*	M	Butler	PA	6.15.2012	9.5428	nocturnal
PA039	*Photinus pyralis*	M	Butler	PA	6.15.2012	11.0322	nocturnal
PA040	*Photinus pyralis*	M	Butler	PA	6.15.2012	14.1119	nocturnal
PA041	*Photinus pyralis*	M	Butler	PA	6.15.2012	6.1771	nocturnal
PA042	*Photinus pyralis*	M	Butler	PA	6.15.2012	16.0237	nocturnal
PA043	*Photinus pyralis*	M	Butler	PA	6.15.2012	13.4633	nocturnal
PA044	*Photinus pyralis*	M	Butler	PA	6.15.2012	13.0497	nocturnal
PA045	*Photinus pyralis*	M	Butler	PA	6.15.2012	13.3645	nocturnal
PA046	*Photinus pyralis*	M	Butler	PA	6.15.2012	11.8305	nocturnal
PA047	*Photinus pyralis*	M	Butler	PA	6.15.2012	10.0378	nocturnal
PA048	*Photinus marginellis*	M	Butler	PA	6.15.2012	4.959	nocturnal
PA049	*Photinus marginellis*	M	Butler	PA	6.15.2012	3.0547	nocturnal
PA050	*Photinus marginellis*	M	Butler	PA	6.15.2012	6.3385	nocturnal
PA051	*Photinus marginellis*	M	Butler	PA	6.15.2012	4.6833	nocturnal
PA052	*Photinus marginellis*	M	Butler	PA	6.15.2012	3.9043	nocturnal
PA053	*Photinus marginellis*	M	Butler	PA	6.15.2012	3.5967	nocturnal
PA054	*Photinus marginellis*	M	Butler	PA	6.15.2012	2.9508	nocturnal
PA055	*Photinus marginellis*	M	Butler	PA	6.15.2012	3.9595	nocturnal
PA056	*Photinus marginellis*	M	Butler	PA	6.15.2012	3.6092	nocturnal
PA057	*Photuris sp.*	M	Butler	PA	6.15.2012	11.2741	diurnal
PA058	*Photuris sp.*	M	Butler	PA	6.15.2012	15.5154	diurnal
PA059	*Photuris sp.*	M	Butler	PA	6.15.2012	7.3529	diurnal
PA060	*Photuris sp.*	F	Butler	PA	6.15.2012	25.1567	diurnal
PA061	*Photuris sp.*	M	Forest	PA	6.16.2012	8.7545	diurnal
PA062	*Photinus carolinus*	M	Forest	PA	6.16.2012	7.7562	nocturnal

Table 3.8: Continued

ID	Species	Sex	County	State	Date Coll.	Dry Wt.	Nocturnal/Diurnal
PA063	*Photinus carolinus*	M	Forest	PA	6.16.2012	6.0917	nocturnal
PA064	*Photinus carolinus*	M	Forest	PA	6.16.2012	9.3131	nocturnal
PA065	*Photinus carolinus*	M	Forest	PA	6.16.2012	5.625	nocturnal
PA066	*Photinus carolinus*	M	Forest	PA	6.16.2012	12.0009	nocturnal
PA067	*Photinus carolinus*	M	Forest	PA	6.16.2012	10.1685	nocturnal
PA068	*Photinus carolinus*	M	Forest	PA	6.16.2012	9.7561	nocturnal
PA069	*Photinus carolinus*	M	Forest	PA	6.16.2012	9.1534	nocturnal
PA070	*Photinus carolinus*	M	Forest	PA	6.16.2012	8.0486	nocturnal
PA071	*Photinus carolinus*	M	Forest	PA	6.16.2012	14.9674	nocturnal
PA072	*Photinus carolinus*	M	Forest	PA	6.16.2012	10.9102	nocturnal
PA073	*Photinus carolinus*	M	Forest	PA	6.16.2012	6.1868	nocturnal
PA074	*Photuris sp.*	F	Forest	PA	6.16.2012	17.7909	diurnal
PA075	*Photuris sp.*	M	Forest	PA	6.16.2012	11.9887	diurnal
PA076	*Photuris sp.*	M	Forest	PA	6.16.2012	13.2139	diurnal
PA077	*Photuris sp.*	M	Forest	PA	6.16.2012	10.0502	diurnal
PA078	*Photuris sp.*	M	Forest	PA	6.16.2012	10.0111	diurnal
PA079	*Photuris sp.*	M	Forest	PA	6.16.2012	7.7094	diurnal
PA080	*Photuris sp.*	M	Forest	PA	6.16.2012	13.2885	diurnal
PA081	*Photuris sp.*	M	Forest	PA	6.16.2012	9.1695	diurnal
PA082	*Photuris sp.*	M	Forest	PA	6.16.2012	14.8093	diurnal
PA083	*Photuris sp.*	M	Forest	PA	6.16.2012	10.6883	diurnal
PA084	*Photuris sp.*	M	Forest	PA	6.16.2012	8.4102	diurnal
PA086	*Photinus carolinus*	M	Forest	PA	6.16.2012	8.3071	nocturnal
PA087	*Photinus carolinus*	M	Forest	PA	6.16.2012	10.7735	nocturnal
PA088	*Photinus macdermotti*	M	Forest	PA	6.17.2012	5.5912	nocturnal
PA089	*Photinus macdermotti*	M	Forest	PA	6.17.2012	5.918	nocturnal
PA090	*Photinus macdermotti*	F	Forest	PA	6.17.2012	7.8829	nocturnal
PA091	*Photinus macdermotti*	M	Forest	PA	6.17.2012	2.2579	nocturnal
PA092	*Photuris sp.*	F	Forest	PA	6.17.2012	18.7225	diurnal
PA093	*Photuris sp.*	M	Forest	PA	6.17.2012	7.7825	diurnal
PA094	*Photuris sp.*	M	Forest	PA	6.17.2012	15.3277	diurnal

Table 3.8: Continued

ID	Species	Sex	County	State	Date Coll.	Dry Wt.	Nocturnal/Diurnal
PA095	*Photuris sp.*	M	Forest	PA	6.17.2012	5.828	diurnal
PA096	*Photuris sp.*	M	Forest	PA	6.17.2012	18.6836	diurnal
PA097	*Photuris sp.*	M	Forest	PA	6.17.2012	14.9495	diurnal
PA098	*Lucidota atra*	M	Forest	PA	6.17.2012	5.1715	diurnal
PA099	*Lucidota atra*	M	Forest	PA	6.17.2012	7.9235	diurnal
PA100	*Lucidota atra*	M	Forest	PA	6.17.2012	7.4018	diurnal
PA101	*Lucidota atra*	M	Forest	PA	6.17.2012	6.1032	diurnal
PA102	*Lucidota atra*	M	Forest	PA	6.17.2012	4.5181	diurnal
PA103	*Lucidota atra*	M	Forest	PA	6.17.2012	2.6809	diurnal
PA104	*Lucidota atra*	M	Forest	PA	6.17.2012	8.5793	diurnal
PA105	*Lucidota atra*	M	Forest	PA	6.17.2012	6.8679	diurnal
PA106	*Lucidota atra*	M	Forest	PA	6.17.2012	7.7612	diurnal
PA107	*Photinus macdermotti*	M	Forest	PA	6.17.2012	6.9115	nocturnal
PA108	*Photinus macdermotti*	M	Forest	PA	6.17.2012	7.2954	nocturnal
PA110	*Lucidota atra*	M	Forest	PA	6.18.2012	3.7526	diurnal
PA111	*Pyropyga decipiens*	F	Forest	PA	6.18.2012	1.775	diurnal
PA112	*Photinus carolinus*	M	Forest	PA	6.18.2012	7.2753	nocturnal
PA113	*Pyropyga decipiens*	F	Forest	PA	6.18.2012	1.9848	diurnal
PA114	*Pyropyga decipiens*	M	Forest	PA	6.18.2012	1.6605	diurnal
PA115	*Lucidota atra*	M	Forest	PA	6.18.2012	4.1504	diurnal
PA117	*Pyropyga decipiens*	F	Forest	PA	6.18.2012	2.3125	diurnal
PA118	*Pyropyga decipiens*	M	Forest	PA	6.18.2012	2.1085	diurnal
PA119	*Pyropyga decipiens*	F	Forest	PA	6.18.2012	2.3056	diurnal
PA120	*Pyropyga decipiens*	F	Forest	PA	6.18.2012	2.2001	diurnal
PA121	*Lucidota atra*	M	Forest	PA	6.18.2012	2.8943	diurnal
PA122	*Pyropyga decipiens*	M	Forest	PA	6.18.2012	2.5689	diurnal
PA123	*Pyropyga decipiens*	F	Forest	PA	6.18.2012	1.7224	diurnal
PA124	*Pyropyga decipiens*	M	Forest	PA	6.18.2012	1.4997	diurnal
PA125	*Pyropyga decipiens*	M	Forest	PA	6.18.2012	1.7203	diurnal
PA126	*Pyropyga decipiens*	M	Forest	PA	6.18.2012	2.4467	diurnal
PA127	*Pyropyga decipiens*	M	Forest	PA	6.18.2012	2.6198	diurnal

Table 3.8: Continued

ID	Species	Sex	County	State	Date Coll.	Dry Wt.	Nocturnal/Diurnal
PA128	*Pyropyga decipiens*	F	Forest	PA	6.18.2012	1.8875	diurnal
PA129	*Pyropyga decipiens*	M	Forest	PA	6.18.2012	1.7869	diurnal
PA130	*Pyropyga decipiens*	M	Forest	PA	6.18.2012	2.0833	diurnal
PA131	*Pyropyga decipiens*	M	Forest	PA	6.18.2012	1.2295	diurnal
PA136	*Lucidota atra*	M	Forest	PA	6.18.2012	5.7537	diurnal
PA137	*Lucidota atra*	M	Forest	PA	6.18.2012	4.7999	diurnal
PA138	*Pyropyga decipiens*	F	Forest	PA	6.18.2012	2.2309	diurnal
PA139	*Pyropyga decipiens*	M	Forest	PA	6.18.2012	1.2811	diurnal
PA140	*Lucidota atra*	M	Forest	PA	6.18.2012	4.4648	diurnal
PA141	*Lucidota atra*	M	Forest	PA	6.18.2012	5.9788	diurnal
PA142	*Lucidota atra*	M	Forest	PA	6.18.2012	3.7722	diurnal
PA143	*Lucidota atra*	M	Forest	PA	6.18.2012	5.7595	diurnal
PA144	*Lucidota atra*	M	Forest	PA	6.18.2012	3.7381	diurnal
PA145	*Lucidota atra*	M	Forest	PA	6.18.2012	6.1971	diurnal
PA146	*Lucidota atra*	M	Forest	PA	6.18.2012	5.4869	diurnal
PA147	*Pyropyga decipiens*	M	Forest	PA	6.18.2012	2.3894	diurnal
PA148	*Pyropyga decipiens*	M	Forest	PA	6.18.2012	1.8863	diurnal
PA149	*Pyropyga decipiens*	M	Forest	PA	6.18.2012	2.6116	diurnal
PA150	*Pyropyga decipiens*	M	Forest	PA	6.18.2012	1.4439	diurnal
PA151	*Pyropyga decipiens*	M	Forest	PA	6.18.2012	2.1474	diurnal
PA152	*Pyropyga decipiens*	M	Forest	PA	6.18.2012	1.8044	diurnal
PA153	*Pyropyga decipiens*	F	Forest	PA	6.18.2012	2.3937	diurnal
PA154	*Pyropyga decipiens*	M	Forest	PA	6.18.2012	1.6809	diurnal
PA156	*Photinus carolinus*	M	Forest	PA	6.18.2012	7.9833	nocturnal
PA157	*Photinus carolinus*	M	Forest	PA	6.18.2012	7.8062	nocturnal
PA159	*Photinus carolinus*	M	Forest	PA	6.18.2012	9.2055	nocturnal
PA160	*Photuris sp.*	M	Forest	PA	6.18.2012	7.59	diurnal
PA161	*Photinus macdermotti*	F	Forest	PA	6.18.2012	10.0229	nocturnal
PA162	*Photuris sp.*	M	Forest	PA	6.18.2012	14.4424	diurnal
PA163	*Photuris sp.*	M	Forest	PA	6.18.2012	13.478	diurnal
PA164	*Photuris sp.*	M	Forest	PA	6.18.2012	9.8165	diurnal

Table 3.8: Continued

ID	Species	Sex	County	State	Date Coll.	Dry Wt.	Nocturnal/Diurnal
PA165	*Photuris sp.*	M	Forest	PA	6.18.2012	10.5474	diurnal
PA167	*Photuris sp.*	M	Forest	PA	6.18.2012	11.5876	diurnal
PA168	*Photuris sp.*	M	Forest	PA	6.18.2012	15.0395	diurnal
PA169	*Photuris sp.*	M	Forest	PA	6.18.2012	12.207	diurnal
PA170	*Photuris sp.*	M	Forest	PA	6.18.2012	10.8069	diurnal
PA171	*Photuris sp.*	M	Forest	PA	6.18.2012	13.4076	diurnal
PA172	*Photuris sp.*	M	Forest	PA	6.18.2012	11.8497	diurnal
PA173	*Photuris sp.*	M	Forest	PA	6.18.2012	11.3715	diurnal
PA174	*Photuris sp.*	M	Forest	PA	6.18.2012	8.2441	diurnal
PA175	*Photinus macdermotti*	M	Forest	PA	6.18.2012	5.8347	nocturnal
PA176	*Photinus macdermotti*	M	Forest	PA	6.18.2012	2.9712	nocturnal
PA177	*Photinus macdermotti*	F	Forest	PA	6.18.2012	4.8432	nocturnal
PA178	*Photinus macdermotti*	M	Forest	PA	6.18.2012	3.4219	nocturnal
PA179	*Photinus macdermotti*	M	Forest	PA	6.18.2012	3.9799	nocturnal
PA180	*Photinus macdermotti*	M	Forest	PA	6.18.2012	5.8532	nocturnal
PA181	*Photinus macdermotti*	M	Forest	PA	6.18.2012	6.7008	nocturnal
PA182	*Photinus macdermotti*	M	Forest	PA	6.18.2012	5.3613	nocturnal
PA183	*Photinus macdermotti*	M	Forest	PA	6.18.2012	4.1346	nocturnal
PA184	*Pyropyga decipiens*	M	Forest	PA	6.18.2012	1.4273	diurnal
PA185	*Pyropyga decipiens*	M	Forest	PA	6.18.2012	2.5545	diurnal
PA186	*Pyropyga decipiens*	F	Forest	PA	6.18.2012	2.6762	diurnal
PA187	*Lucidota punctata*	M	Forest	PA	6.19.2012	2.142	diurnal
PA188	*Lucidota punctata*	M	Forest	PA	6.19.2012	2.3	diurnal
PA189	*Lucidota punctata*	M	Forest	PA	6.19.2012	1.9258	diurnal
PA190	*Lucidota punctata*	M	Forest	PA	6.19.2012	1.7916	diurnal
PA191	*Lucidota punctata*	M	Forest	PA	6.19.2012	1.4755	diurnal
PA192	*Pyropyga decipiens*	F	Forest	PA	6.19.2012	2.6201	diurnal
PA193	*Pyropyga decipiens*	M	Forest	PA	6.19.2012	1.1145	diurnal
PA194	*Pyropyga decipiens*	F	Forest	PA	6.19.2012	1.1145	diurnal
PA195	*Pyropyga decipiens*	F	Forest	PA	6.19.2012	1.9866	diurnal
PA196	*Pyropyga decipiens*	M	Forest	PA	6.19.2012	1.6701	diurnal

Table 3.8: Continued

ID	Species	Sex	County	State	Date Coll.	Dry Wt.	Nocturnal/Diurnal
PA197	*Pyropyga decipiens*	F	Forest	PA	6.19.2012	1.7523	diurnal
PA198	*Pyropyga decipiens*	F	Forest	PA	6.19.2012	3.2811	diurnal
PA199	*Pyropyga decipiens*	M	Forest	PA	6.19.2012	2.2884	diurnal
PA200	*Pyropyga decipiens*	F	Forest	PA	6.19.2012	2.8529	diurnal
PA201	*Pyropyga decipiens*	M	Forest	PA	6.19.2012	1.4186	diurnal
PA202	*Pyropyga decipiens*	M	Forest	PA	6.19.2012	2.0024	diurnal
PA203	*Pyropyga decipiens*	F	Forest	PA	6.19.2012	2.2513	diurnal
PA204	*Lucidota atra*	M	Forest	PA	6.19.2012	6.6556	diurnal
PA205	*Photuris sp.*	F	Forest	PA	6.19.2012	26.2841	diurnal
PA206	*Photuris sp.*	F	Forest	PA	6.19.2012	8.3674	diurnal
PA207	*Photuris sp.*	M	Forest	PA	6.19.2012	13.0766	diurnal
PA208	*Photuris sp.*	M	Forest	PA	6.19.2012	11.9924	diurnal
PA209	*Photuris sp.*	F	Forest	PA	6.19.2012	15.009	diurnal
PA211	*Photuris sp.*	M	Forest	PA	6.19.2012	8.318	diurnal
PA212	*Photinus carolinus*	M	Forest	PA	6.19.2012	10.2538	nocturnal
PA213	*Photinus carolinus*	M	Forest	PA	6.19.2012	9.1603	nocturnal
PA214	*Photinus carolinus*	M	Forest	PA	6.19.2012	8.1254	nocturnal
PA215	*Photinus carolinus*	M	Forest	PA	6.19.2012	7.2149	nocturnal
PA216	*Photinus carolinus*	M	Forest	PA	6.19.2012	12.1811	nocturnal
PA217	*Photinus carolinus*	M	Forest	PA	6.19.2012	7.9563	nocturnal
PA218	*Photinus carolinus*	M	Forest	PA	6.19.2012	7.8	nocturnal
PA219	*Photinus carolinus*	M	Forest	PA	6.19.2012	5.9515	nocturnal
PA220	*Photinus carolinus*	M	Forest	PA	6.19.2012	10.8898	nocturnal
PA221	*Photinus carolinus*	M	Forest	PA	6.19.2012	8.8501	nocturnal
PA222	*Photinus carolinus*	M	Forest	PA	6.19.2012	8.6529	nocturnal
PA223	*Photinus carolinus*	M	Forest	PA	6.19.2012	5.4487	nocturnal
PA224	*Photinus carolinus*	M	Forest	PA	6.19.2012	9.0684	nocturnal
PA225	*Photinus carolinus*	M	Forest	PA	6.19.2012	6.3398	nocturnal
PA226	*Photinus carolinus*	M	Forest	PA	6.19.2012	6.7442	nocturnal
PA227	*Photinus carolinus*	M	Forest	PA	6.19.2012	7.8557	nocturnal
PA228	*Photinus carolinus*	M	Forest	PA	6.19.2012	9.9085	nocturnal

Table 3.8: Continued

ID	Species	Sex	County	State	Date Coll.	Dry Wt.	Nocturnal/Diurnal
PA229	*Photinus carolinus*	M	Forest	PA	6.19.2012	9.8682	nocturnal
PA230	*Photinus carolinus*	M	Forest	PA	6.19.2012	6.8485	nocturnal
PA231	*Photinus carolinus*	M	Forest	PA	6.19.2012	9.979	nocturnal
PA232	*Photinus carolinus*	M	Forest	PA	6.19.2012	8.9891	nocturnal
PA233	*Photinus carolinus*	M	Forest	PA	6.19.2012	6.6175	nocturnal
PA234	*Photinus carolinus*	M	Forest	PA	6.19.2012	9.779	nocturnal
PA235	*Photinus carolinus*	M	Forest	PA	6.19.2012	4.3368	nocturnal
PA236	*Lucidota atra*	F	Forest	PA	6.20.2012	20.2291	diurnal
PA237	*Photuris sp.*	M	Forest	PA	6.20.2012	10.6383	diurnal
PA238	*Photuris sp.*	F	Forest	PA	6.20.2012	19.7594	diurnal
PA239	*Photuris sp.*	M	Forest	PA	6.20.2012	10.8569	diurnal
PA240	*Photuris sp.*	M	Forest	PA	6.20.2012	14.2324	diurnal
PA241	*Photuris sp.*	M	Forest	PA	6.20.2012	10.2462	diurnal
PA242	*Photuris sp.*	M	Forest	PA	6.20.2012	10.2477	diurnal
PA243	*Photuris sp.*	M	Forest	PA	6.20.2012	16.5175	diurnal
PA244	*Photuris sp.*	F	Forest	PA	6.20.2012	35.8769	diurnal
PA245	*Photuris sp.*	F	Forest	PA	6.20.2012	36.6955	diurnal
PA246	*Photuris sp.*	M	Forest	PA	6.20.2012	18.4935	diurnal
PA247	*Photuris sp.*	M	Forest	PA	6.20.2012	13.9799	diurnal
PA248	*Photuris sp.*	M	Forest	PA	6.20.2012	13.6202	diurnal
PA249	*Photuris sp.*	M	Forest	PA	6.20.2012	9.9619	diurnal
PA250	*Photuris sp.*	M	Forest	PA	6.20.2012	13.6869	diurnal
PA251	*Photuris sp.*	M	Forest	PA	6.20.2012	13.583	diurnal
PA252	*Photuris sp.*	M	Forest	PA	6.20.2012	11.0454	diurnal
PA253	*Photuris sp.*	M	Forest	PA	6.20.2012	15.6406	diurnal
PA254	*Photuris sp.*	M	Forest	PA	6.20.2012	13.7825	diurnal
PA255	*Photuris sp.*	M	Forest	PA	6.20.2012	12.7728	diurnal
PA256	*Photuris sp.*	M	Forest	PA	6.20.2012	18.0452	diurnal
PA257	*Photuris sp.*	M	Forest	PA	6.20.2012	16.6938	diurnal
PA258	*Photuris sp.*	M	Forest	PA	6.20.2012	15.2419	diurnal
PA259	*Photuris sp.*	M	Forest	PA	6.20.2012	14.4244	diurnal

Table 3.8: Continued

ID	Species	Sex	County	State	Date Coll.	Dry Wt.	Nocturnal/Diurnal
PA260	*Photuris sp.*	F	Forest	PA	6.20.2012	22.1215	diurnal
PA261	*Photuris sp.*	M	Forest	PA	6.20.2012	12.738	diurnal
PA262	*Photinus carolinus*	M	Forest	PA	6.20.2012	9.2444	nocturnal
PA263	*Photinus carolinus*	M	Forest	PA	6.20.2012	9.1782	nocturnal
PA264	*Photinus carolinus*	M	Forest	PA	6.20.2012	7.6396	nocturnal
PA265	*Photinus carolinus*	M	Forest	PA	6.20.2012	6.5064	nocturnal
PA266	*Photinus carolinus*	M	Forest	PA	6.20.2012	7.1175	nocturnal
PA267	*Photinus macdermotti*	M	Forest	PA	6.20.2012	4.0525	nocturnal
PA268	*Photinus macdermotti*	M	Forest	PA	6.20.2012	5.9748	nocturnal
PA269	*Photinus macdermotti*	M	Forest	PA	6.20.2012	3.7399	nocturnal
PA270	*Photinus macdermotti*	M	Forest	PA	6.20.2012	4.6152	nocturnal
PA271	*Photinus marginellis*	M	Forest	PA	6.20.2012	2.9096	nocturnal
PA272	*Photinus carolinus*	M	Forest	PA	6.19.2012	7.6909	nocturnal
PA273	*Photinus carolinus*	M	Forest	PA	6.19.2012	7.8992	nocturnal
PA274	*Photinus carolinus*	M	Forest	PA	6.19.2012	8.2228	nocturnal
PA275	*Photinus carolinus*	M	Forest	PA	6.19.2012	10.2604	nocturnal
PA276	*Photinus macdermotti*	F	Forest	PA	6.21.2012	7.343	nocturnal
PA277	*Photinus macdermotti*	M	Forest	PA	6.21.2012	5.7788	nocturnal
PA278	*Photinus macdermotti*	M	Forest	PA	6.21.2012	5.9818	nocturnal
PA279	*Photinus macdermotti*	M	Forest	PA	6.21.2012	4.546	nocturnal
PA280	*Photinus macdermotti*	M	Forest	PA	6.21.2012	6.5403	nocturnal
PA281	*Photinus macdermotti*	M	Forest	PA	6.21.2012	4.3849	nocturnal
PA282	*Photinus macdermotti*	M	Forest	PA	6.21.2012	4.3261	nocturnal
PA283	*Photinus macdermotti*	M	Forest	PA	6.21.2012	5.9827	nocturnal
PA284	*Photinus macdermotti*	M	Forest	PA	6.21.2012	5.3646	nocturnal
PA285	*Photinus macdermotti*	M	Forest	PA	6.21.2012	4.2798	nocturnal
PA286	*Photinus carolinus*	M	Forest	PA	6.21.2012	9.4755	nocturnal
PA288	*Photuris sp.*	M	Forest	PA	6.21.2012	7.8848	diurnal
PA289	*Photinus carolinus*	M	Forest	PA	6.21.2012	6.1933	nocturnal
PA290	*Photinus carolinus*	M	Forest	PA	6.21.2012	4.4636	nocturnal
PA291	*Photinus carolinus*	F	Forest	PA	6.21.2012	9.7345	nocturnal

Table 3.8: Continued

ID	Species	Sex	County	State	Date Coll.	Dry Wt.	Nocturnal/Diurnal
PA292	*Photinus carolinus*	M	Forest	PA	6.21.2012	8.2045	nocturnal
PA293	*Photinus carolinus*	M	Forest	PA	6.21.2012	8.282	nocturnal
PA294	*Lucidota atra*	M	Forest	PA	6.21.2012	5.3631	diurnal
PA295	*Lucidota atra*	M	Forest	PA	6.21.2012	6.0015	diurnal
PA296	*Lucidota atra*	M	Forest	PA	6.21.2012	5.4321	diurnal
PA297	*Pyropyga decipiens*	M	Forest	PA	6.21.2012	1.7481	diurnal
PA298	*Pyropyga decipiens*	M	Forest	PA	6.21.2012	1.7045	diurnal
PA299	*Pyropyga decipiens*	F	Forest	PA	6.21.2012	3.1823	diurnal
PA300	*Pyropyga decipiens*	F	Forest	PA	6.21.2012	2.3022	diurnal
PA301	*Pyropyga decipiens*	F	Forest	PA	6.21.2012	2.9161	diurnal
PA302	*Pyropyga decipiens*	F	Forest	PA	6.21.2012	2.1414	diurnal
PA303	*Pyropyga decipiens*	F	Forest	PA	6.21.2012	1.7783	diurnal
PA304	*Pyropyga decipiens*	M	Forest	PA	6.21.2012	2.1889	diurnal
PA305	*Pyropyga decipiens*	M	Forest	PA	6.21.2012	1.2339	diurnal
PA307	*Photuris sp.*	M	Forest	PA	6.16.2012	16.5574	diurnal
PA308	*Photuris sp.*	M	Forest	PA	6.16.2012	10.3437	diurnal
PA309	*Photuris sp.*	M	Forest	PA	6.16.2012	14.5725	diurnal
PA310	*Photinus carolinus*	M	Forest	PA	6.16.2012	4.8497	nocturnal
PA311	*Photuris sp.*	M	Forest	PA	6.16.2012	13.7296	diurnal
PA312	*Photuris sp.*	M	Forest	PA	6.16.2012	13.3924	diurnal
PA313	*Photuris sp.*	M	Forest	PA	6.16.2012	15.1067	diurnal
PA314	*Photuris sp.*	M	Forest	PA	6.16.2012	21.3017	diurnal
PA315	*Photuris sp.*	M	Forest	PA	6.16.2012	16.0156	diurnal
PA316	*Photuris sp.*	M	Forest	PA	6.16.2012	14.9798	diurnal
PA317	*Photuris sp.*	M	Forest	PA	6.16.2012	13.4572	diurnal
PA318	*Photuris sp.*	M	Forest	PA	6.16.2012	15.611	diurnal
PA319	*Photuris sp.*	M	Forest	PA	6.16.2012	16.7679	diurnal
PA320	*Photinus macdermotti*	M	Forest	PA	6.16.2012	5.2185	nocturnal
PA321	*Photinus macdermotti*	M	Forest	PA	6.16.2012	7.0356	nocturnal
PA322	*Photinus macdermotti*	M	Forest	PA	6.16.2012	5.9815	nocturnal
PA323	*Photinus macdermotti*	M	Forest	PA	6.16.2012	3.8109	nocturnal

Table 3.8: Continued

ID	Species	Sex	County	State	Date Coll.	Dry Wt.	Nocturnal/Diurnal
PA324	*Photinus macdermotti*	M	Forest	PA	6.16.2012	7.3513	nocturnal
PA325	*Photinus macdermotti*	M	Forest	PA	6.16.2012	7.1408	nocturnal
PA326	*Photinus macdermotti*	M	Forest	PA	6.16.2012	5.5172	nocturnal
PA327	*Photinus carolinus*	M	Forest	PA	6.16.2012	9.834	nocturnal
PA328	*Photinus carolinus*	M	Forest	PA	6.16.2012	10.6758	nocturnal
PA329	*Photinus carolinus*	M	Forest	PA	6.16.2012	7.9513	nocturnal
PA330	*Photinus carolinus*	M	Forest	PA	6.16.2012	9.0467	nocturnal
PA331	*Photuris sp.*	F	Forest	PA	6.16.2012	20.6756	diurnal
PA332	*Photuris sp.*	F	Forest	PA	6.16.2012	28.8446	diurnal
PA333	*Lucidota atra*	M	Forest	PA	6.16.2012	4.4693	diurnal
PA335	*Lucidota atra*	F	Forest	PA	6.16.2012	6.6984	diurnal
PA336	*Pyropyga decipiens*	F	Forest	PA	6.21.2012	3.2615	diurnal
PA337	*Photuris sp.*	M	Forest	PA	6.22.2012	10.6802	diurnal
PA338	*Photuris sp.*	M	Forest	PA	6.22.2012	16.6916	diurnal
PA339	*Photuris sp.*	F	Forest	PA	6.22.2012	17.9528	diurnal
PA340	*Photuris sp.*	M	Forest	PA	6.22.2012	11.1404	diurnal
PA341	*Photuris sp.*	M	Forest	PA	6.22.2012	11.2204	diurnal
PA342	*Photuris sp.*	M	Forest	PA	6.22.2012	12.3075	diurnal
PA343	*Photuris sp.*	F	Forest	PA	6.22.2012	12.3452	diurnal
PA344	*Photuris sp.*	M	Forest	PA	6.22.2012	13.1739	diurnal
PA345	*Photuris sp.*	M	Forest	PA	6.22.2012	13.8285	diurnal
PA346	*Photuris sp.*	M	Forest	PA	6.22.2012	13.7414	diurnal
PA347	*Photuris sp.*	M	Forest	PA	6.22.2012	13.4631	diurnal
PA348	*Pyropyga decipiens*	M	Forest	PA	6.22.2012	2.1361	diurnal
PA349	*Lucidota atra*	M	Forest	PA	6.22.2012	3.7766	diurnal
PA350	*Pyropyga decipiens*	F	Forest	PA	6.22.2012	2.6778	diurnal
PA351	*Photinus carolinus*	M	Forest	PA	6.22.2012	8.2042	nocturnal
PA352	*Photinus carolinus*	M	Forest	PA	6.22.2012	6.129	nocturnal
PA353	*Photinus carolinus*	M	Forest	PA	6.22.2012	7.5776	nocturnal
PA354	*Photinus carolinus*	F	Forest	PA	6.22.2012	13.6134	nocturnal
PA355	*Photinus carolinus*	M	Forest	PA	6.22.2012	10.606	nocturnal

Table 3.8: Continued

ID	Species	Sex	County	State	Date Coll.	Dry Wt.	Nocturnal/Diurnal
PA356	*Photinus carolinus*	M	Forest	PA	6.22.2012	6.9179	nocturnal
PA357	*Photinus carolinus*	M	Forest	PA	6.22.2012	11.7701	nocturnal
PA358	*Photinus obscurellus*	F	Forest	PA	6.22.2012	4.8331	nocturnal
PA359	*Photinus obscurellus*	M	Forest	PA	6.22.2012	2.9141	nocturnal
PA360	*Photinus obscurellus*	M	Forest	PA	6.22.2012	3.406	nocturnal
PA361	*Photinus obscurellus*	M	Forest	PA	6.22.2012	3.8962	nocturnal
PA362	*Photinus obscurellus*	F	Forest	PA	6.22.2012	2.5913	nocturnal
PA363	*Photinus obscurellus*	M	Forest	PA	6.22.2012	4.0216	nocturnal
PA364	*Photinus obscurellus*	M	Forest	PA	6.22.2012	5.4816	nocturnal
PA365	*Photinus obscurellus*	M	Forest	PA	6.22.2012	4.0408	nocturnal
PA366	*Photinus obscurellus*	M	Forest	PA	6.22.2012	4.5228	nocturnal
PA367	*Photinus obscurellus*	M	Forest	PA	6.22.2012	3.8763	nocturnal
PA368	*Photinus obscurellus*	M	Forest	PA	6.22.2012	6.1945	nocturnal
PA369	*Photinus obscurellus*	M	Forest	PA	6.22.2012	3.7599	nocturnal
PA370	*Photinus obscurellus*	M	Forest	PA	6.22.2012	2.5245	nocturnal
PA371	*Photinus obscurellus*	M	Forest	PA	6.22.2012	3.3365	nocturnal
PA372	*Photinus obscurellus*	M	Forest	PA	6.22.2012	2.1293	nocturnal
PA373	*Photinus obscurellus*	M	Forest	PA	6.22.2012	3.0775	nocturnal
PA374	*Lucidota atra*	Egg	Butler	PA	6.22.2012	0.0577	diurnal
PA377	*Photuris sp.*	Egg	Forest	PA	6.18.2012	0.0851	diurnal
PA379	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.0749	nocturnal
PA380	*Photinus carolinus*	M	Forest	PA	6.18.2012	7.0918	nocturnal
PA381	*Photinus carolinus*	F	Forest	PA	6.18.2012	5.0709	nocturnal
PA382	*Photinus pyralis*	M	Berks	PA	6.24.2012	12.6091	nocturnal
PA383	*Photinus pyralis*	M	Berks	PA	6.24.2012	12.0668	nocturnal
PA384	*Photinus pyralis*	M	Berks	PA	6.24.2012	10.5099	nocturnal
PA385	*Photinus pyralis*	M	Berks	PA	6.24.2012	17.8992	nocturnal
PA386	*Photinus pyralis*	M	Berks	PA	6.24.2012	8.2786	nocturnal
PA387	*Photinus pyralis*	M	Berks	PA	6.24.2012	10.2591	nocturnal
PA388	*Photinus pyralis*	M	Berks	PA	6.24.2012	13.2838	nocturnal
PA389	*Photinus pyralis*	M	Berks	PA	6.24.2012	10.9307	nocturnal

Table 3.8: Continued

ID	Species	Sex	County	State	Date Coll.	Dry Wt.	Nocturnal/Diurnal
PA390	*Photinus pyralis*	M	Berks	PA	6.24.2012	11.1318	nocturnal
PA391	*Photinus pyralis*	M	Berks	PA	6.24.2012	15.2206	nocturnal
PA392	*Photinus pyralis*	M	Berks	PA	6.24.2012	7.4008	nocturnal
PA393	*Photinus pyralis*	M	Berks	PA	6.24.2012	7.0559	nocturnal
PA394	*Photinus pyralis*	M	Berks	PA	6.24.2012	10.0262	nocturnal
PA395	*Photinus pyralis*	M	Berks	PA	6.24.2012	13.3241	nocturnal
PA396	*Photinus pyralis*	M	Berks	PA	6.24.2012	13.8274	nocturnal
PA397	*Photinus pyralis*	M	Berks	PA	6.24.2012	10.3179	nocturnal
PA398	*Photinus pyralis*	M	Berks	PA	6.24.2012	17.2041	nocturnal
PA399	*Photinus pyralis*	M	Berks	PA	6.24.2012	15.1009	nocturnal
PA400	*Photinus pyralis*	M	Berks	PA	6.24.2012	10.0362	nocturnal
PA401	*Photinus pyralis*	M	Berks	PA	6.24.2012	10.7074	nocturnal
PA402	*Photinus pyralis*	M	Berks	PA	6.24.2012	13.9781	nocturnal
PA403	*Photinus pyralis*	M	Berks	PA	6.24.2012	15.4613	nocturnal
PA404	*Photinus pyralis*	M	Berks	PA	6.24.2012	4.8463	nocturnal
PA405	*Photinus pyralis*	M	Berks	PA	6.24.2012	9.4526	nocturnal
PA406	*Photinus pyralis*	M	Berks	PA	6.24.2012	11.9854	nocturnal
PA407	*Photinus pyralis*	M	Berks	PA	6.24.2012	10.3202	nocturnal
PA408	*Photinus pyralis*	M	Berks	PA	6.24.2012	7.2701	nocturnal
PA409	*Photinus pyralis*	M	Berks	PA	6.24.2012	10.6817	nocturnal
PA410	*Photinus pyralis*	M	Berks	PA	6.24.2012	9.4514	nocturnal
PA411	*Photinus pyralis*	M	Berks	PA	6.24.2012	9.044	nocturnal
PA412	*Pyropyga decipiens*	Egg	Forest	PA	6.23.2012	0.0195	diurnal
PA413	*Photuris sp.*	Egg	Forest	PA	6.23.2012	0.0864	diurnal
PA414	*Photuris sp.*	Egg	Forest	PA	6.19.2012	0.0727	diurnal
PA415	*Photinus pyralis*	M	Berks	PA	6.24.2012	8.8438	nocturnal
PA416	*Lucidota atra*	F	Forest	PA	6.24.2012	6.6921	diurnal
PA417	*Lucidota atra*	F	Forest	PA	6.18.2012	14.7723	diurnal
PA418	*Lucidota atra*	Egg	Forest	PA	6.21.2012	0.3045	diurnal
PA419	*Lucidota atra*	Egg	Forest	PA	6.21.2012	0.0822	diurnal
PA420	*Lucidota atra*	Egg	Forest	PA	6.21.2012	0.0868	diurnal

Table 3.8: Continued

ID	Species	Sex	County	State	Date Coll.	Dry Wt.	Nocturnal/Diurnal
PA421	*Lucidota atra*	Egg	Forest	PA	6.21.2012	0.0817	diurnal
PA422	*Lucidota atra*	Egg	Forest	PA	6.21.2012	0.0956	diurnal
PA423	*Lucidota atra*	Egg	Forest	PA	6.21.2012	0.1057	diurnal
PA424	*Lucidota atra*	Egg	Forest	PA	6.21.2012	0.0406	diurnal
PA425	*Lucidota atra*	Egg	Forest	PA	6.21.2012	0.0668	diurnal
PA426	*Lucidota atra*	Egg	Forest	PA	6.21.2012	0.0846	diurnal
PA427	*Lucidota atra*	Egg	Forest	PA	6.21.2012	0.101	diurnal
PA428	*Lucidota atra*	Egg	Forest	PA	6.21.2012	0.1182	diurnal
PA429	*Lucidota atra*	Egg	Forest	PA	6.21.2012	0.0897	diurnal
PA434	*Lucidota atra*	Egg	Butler	PA	6.22.2012	0.0563	diurnal
PA435	*Lucidota atra*	Egg	Butler	PA	6.22.2012	0.0575	diurnal
PA436	*Lucidota atra*	Egg	Butler	PA	6.22.2012	0.0588	diurnal
PA437	*Lucidota atra*	Egg	Butler	PA	6.22.2012	0.6455	diurnal
PA438	*Lucidota atra*	Egg	Butler	PA	6.22.2012	0.0563	diurnal
PA439	*Lucidota atra*	Egg	Butler	PA	6.22.2012	0.0474	diurnal
PA440	*Lucidota atra*	Egg	Butler	PA	6.22.2012	0.0635	diurnal
PA441	*Lucidota atra*	Egg	Butler	PA	6.22.2012	0.0557	diurnal
PA442	*Lucidota atra*	Egg	Butler	PA	6.22.2012	0.0605	diurnal
PA444	*Lucidota atra*	Egg	Butler	PA	6.22.2012	0.0551	diurnal
PA445	*Lucidota atra*	Egg	Butler	PA	6.22.2012	0.056	diurnal
PA446	*Lucidota atra*	Egg	Butler	PA	6.22.2012	0.0598	diurnal
PA447	*Lucidota atra*	Egg	Butler	PA	6.22.2012	0.067	diurnal
PA448	*Lucidota atra*	Egg	Butler	PA	6.22.2012	0.0633	diurnal
PA449	*Lucidota atra*	Egg	Butler	PA	6.22.2012	0.0594	diurnal
PA450	*Lucidota atra*	Egg	Butler	PA	6.22.2012	0.0231	diurnal
PA451	*Lucidota atra*	Egg	Butler	PA	6.22.2012	0.0551	diurnal
PA452	*Lucidota atra*	Egg	Butler	PA	6.22.2012	0.056	diurnal
PA453	*Lucidota atra*	Egg	Butler	PA	6.22.2012	0.1215	diurnal
PA454	*Lucidota atra*	Egg	Butler	PA	6.22.2012	0.06	diurnal
PA455	*Lucidota atra*	Egg	Butler	PA	6.22.2012	0.0737	diurnal
PA456	*Lucidota atra*	Egg	Butler	PA	6.22.2012	0.0552	diurnal

Table 3.8: Continued

ID	Species	Sex	County	State	Date Coll.	Dry Wt.	Nocturnal/Diurnal
PA457	*Lucidota atra*	Egg	Forest	PA	6.17.2012	0.0814	diurnal
PA458	*Lucidota atra*	Egg	Forest	PA	6.17.2012	0.0619	diurnal
PA459	*Lucidota atra*	Egg	Forest	PA	6.17.2012	0.0584	diurnal
PA460	*Lucidota atra*	Egg	Forest	PA	6.17.2012	0.0571	diurnal
PA461	*Lucidota atra*	Egg	Forest	PA	6.17.2012	0.0588	diurnal
PA462	*Lucidota atra*	Egg	Forest	PA	6.17.2012	0.0595	diurnal
PA463	*Lucidota atra*	Egg	Forest	PA	6.17.2012	0.0665	diurnal
PA464	*Lucidota atra*	Egg	Forest	PA	6.17.2012	0.0622	diurnal
PA465	*Lucidota atra*	Egg	Forest	PA	6.17.2012	0.058	diurnal
PA466	*Lucidota atra*	Egg	Forest	PA	6.17.2012	0.0595	diurnal
PA467	*Lucidota atra*	Egg	Forest	PA	6.17.2012	0.0629	diurnal
PA468	*Lucidota atra*	Egg	Forest	PA	6.17.2012	0.0587	diurnal
PA469	*Lucidota atra*	Egg	Forest	PA	6.17.2012	0.0569	diurnal
PA470	*Lucidota atra*	Egg	Forest	PA	6.17.2012	0.0543	diurnal
PA471	*Photuris sp.*	Egg	Forest	PA	6.18.2012	0.0584	diurnal
PA472	*Photuris sp.*	Egg	Forest	PA	6.18.2012	0.16	diurnal
PA473	*Photuris sp.*	Egg	Forest	PA	6.18.2012	0.0796	diurnal
PA474	*Photuris sp.*	Egg	Forest	PA	6.18.2012	0.0531	diurnal
PA475	*Photuris sp.*	Egg	Forest	PA	6.18.2012	0.0884	diurnal
PA476	*Photuris sp.*	Egg	Forest	PA	6.18.2012	0.1004	diurnal
PA477	*Photuris sp.*	Egg	Forest	PA	6.18.2012	0.0758	diurnal
PA478	*Photuris sp.*	Egg	Forest	PA	6.18.2012	0.0675	diurnal
PA479	*Photuris sp.*	Egg	Forest	PA	6.18.2012	0.0922	diurnal
PA481	*Photuris sp.*	Egg	Forest	PA	6.18.2012	0.0814	diurnal
PA482	*Photuris sp.*	Egg	Forest	PA	6.18.2012	0.0464	diurnal
PA483	*Photuris sp.*	Egg	Forest	PA	6.18.2012	0.0876	diurnal
PA484	*Photuris sp.*	Egg	Forest	PA	6.18.2012	0.0818	diurnal
PA485	*Photuris sp.*	Egg	Forest	PA	6.18.2012	0.07	diurnal
PA486	*Photuris sp.*	Egg	Forest	PA	6.18.2012	0.0925	diurnal
PA487	*Photuris sp.*	Egg	Forest	PA	6.18.2012	0.0821	diurnal
PA488	*Photuris sp.*	Egg	Forest	PA	6.18.2012	0.0777	diurnal

Table 3.8: Continued

ID	Species	Sex	County	State	Date Coll.	Dry Wt.	Nocturnal/Diurnal
PA489	*Photuris sp.*	Egg	Forest	PA	6.18.2012	0.0675	diurnal
PA491	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.0972	nocturnal
PA492	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.0759	nocturnal
PA493	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.1059	nocturnal
PA494	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.0991	nocturnal
PA495	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.086	nocturnal
PA496	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.0899	nocturnal
PA497	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.0737	nocturnal
PA498	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.064	nocturnal
PA499	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.0964	nocturnal
PA500	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.1044	nocturnal
PA501	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.0857	nocturnal
PA502	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.0864	nocturnal
PA503	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.0819	nocturnal
PA504	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.073	nocturnal
PA505	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.0935	nocturnal
PA506	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.0954	nocturnal
PA507	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.1019	nocturnal
PA509	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.0964	nocturnal
PA510	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.181	nocturnal
PA511	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.0735	nocturnal
PA512	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.0948	nocturnal
PA513	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.0834	nocturnal
PA514	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.253	nocturnal
PA515	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.0987	nocturnal
PA516	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.0702	nocturnal
PA517	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.0761	nocturnal
PA518	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.072	nocturnal
PA519	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.0945	nocturnal
PA520	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.0951	nocturnal
PA521	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.0795	nocturnal

Table 3.8: Continued

ID	Species	Sex	County	State	Date Coll.	Dry Wt.	Nocturnal/Diurnal
PA522	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.078	nocturnal
PA523	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.1781	nocturnal
PA524	*Photinus carolinus*	Egg	Forest	PA	6.23.2012	0.185	nocturnal
PA525	*Photuris sp.*	Egg	Forest	PA	6.23.2012	0.0721	diurnal
PA526	*Photuris sp.*	Egg	Forest	PA	6.23.2012	0.0783	diurnal
PA527	*Photuris sp.*	Egg	Forest	PA	6.23.2012	0.0812	diurnal
PA528	*Photuris sp.*	Egg	Forest	PA	6.23.2012	0.0853	diurnal
PA529	*Photuris sp.*	Egg	Forest	PA	6.19.2012	0.0651	diurnal
PA530	*Photuris sp.*	Egg	Forest	PA	6.19.2012	0.0566	diurnal
PA531	*Photuris sp.*	Egg	Forest	PA	6.19.2012	0.0414	diurnal
PA532	*Photuris sp.*	Egg	Forest	PA	6.19.2012	0.0619	diurnal
PA533	*Photuris sp.*	Egg	Forest	PA	6.19.2012	0.0761	diurnal
PA534	*Photuris sp.*	Egg	Forest	PA	6.19.2012	0.0608	diurnal
PA535	*Photuris sp.*	Egg	Forest	PA	6.19.2012	0.0629	diurnal
PA536	*Photuris sp.*	Egg	Forest	PA	6.19.2012	0.0795	diurnal
PA537	*Photuris sp.*	Egg	Forest	PA	6.19.2012	0.072	diurnal
PA538	*Photuris sp.*	Egg	Forest	PA	6.19.2012	0.0717	diurnal
PA539	*Photuris sp.*	Egg	Forest	PA	6.19.2012	0.0729	diurnal
PA540	*Photuris sp.*	Egg	Forest	PA	6.19.2012	0.0722	diurnal
PA541	*Photuris sp.*	Egg	Forest	PA	6.19.2012	0.0353	diurnal
PA542	*Photuris sp.*	Egg	Forest	PA	6.19.2012	0.0718	diurnal
PA543	*Photuris sp.*	Egg	Forest	PA	6.19.2012	0.0763	diurnal
PA544	*Photuris sp.*	Egg	Forest	PA	6.19.2012	0.0587	diurnal
PA545	*Photuris sp.*	Egg	Forest	PA	6.19.2012	0.0778	diurnal
PA546	*Photuris sp.*	Egg	Forest	PA	6.19.2012	0.0689	diurnal
PA547	*Photuris sp.*	Egg	Forest	PA	6.19.2012	0.0518	diurnal
PA548	*Photuris sp.*	Egg	Forest	PA	6.19.2012	0.0767	diurnal
PA549	*Photuris sp.*	Egg	Forest	PA	6.19.2012	0.0673	diurnal
PA550	*Photuris sp.*	Egg	Forest	PA	6.19.2012	0.0807	diurnal
PA551	*Photuris sp.*	Egg	Forest	PA	6.19.2012	0.0728	diurnal
PA552	*Photuris sp.*	Egg	Forest	PA	6.19.2012	0.0695	diurnal

Table 3.8: Continued

ID	Species	Sex	County	State	Date Coll.	Dry Wt.	Nocturnal/Diurnal
PA553	*Photuris sp.*	Egg	Forest	PA	6.19.2012	0.0839	diurnal
PA554	*Photuris sp.*	Egg	Forest	PA	6.19.2012	0.238	diurnal
PA555	*Photuris sp.*	Egg	Forest	PA	6.19.2012	0.0784	diurnal
ZHM001	*Photinus pyralis*	F	Blount	TN	5.17.2012	27.396	nocturnal
ZHM003	*Photinus pyralis*	M	Blount	TN	5.17.2012	11.9933	nocturnal
ZHM004	*Photuris sp.*	M	Blount	TN	5.17.2012	11.2859	diurnal
ZHM005	*Photinus pyralis*	F	Blount	TN	5.17.2012	25.2591	nocturnal
ZHM006	*Photinus pyralis*	M	Blount	TN	5.17.2012	18.8888	nocturnal
ZHM007	*Photuris sp.*	M	Blount	TN	5.17.2012	8.1289	diurnal
ZHM008	*Photuris sp.*	M	Blount	TN	5.17.2012	8.0834	diurnal
ZHM009	*Photuris sp.*	M	Blount	TN	5.17.2012	9.3023	diurnal
ZHM010	*Photuris sp.*	M	Blount	TN	5.17.2012	9.4659	diurnal
ZHM011	*Photuris sp.*	M	Blount	TN	5.17.2012	11.2684	diurnal
ZHM012	*Photuris sp.*	M	Blount	TN	5.17.2012	8.9044	diurnal
ZHM013	*Photuris sp.*	M	Blount	TN	5.17.2012	11.977	diurnal
ZHM014	*Photuris sp.*	M	Blount	TN	5.17.2012	7.4031	diurnal
ZHM015	*Photinus pyralis*	M	Blount	TN	5.17.2012	13.9299	nocturnal
ZHM016	*Photuris sp.*	M	Blount	TN	5.17.2012	12.2333	diurnal
ZHM017	*Photuris sp.*	M	Blount	TN	5.17.2012	9.321	diurnal
ZHM018	*Photuris sp.*	M	Blount	TN	5.17.2012	10.1573	diurnal
ZHM020	*Photuris sp.*	M	Blount	TN	5.17.2012	8.309	diurnal
ZHM021	*Photuris sp.*	M	Blount	TN	5.17.2012	8.2098	diurnal
ZHM022	*Photuris sp.*	M	Blount	TN	5.17.2012	19.1309	diurnal
ZHM023	*Photuris sp.*	F	Blount	TN	5.17.2012	38.0939	diurnal
ZHM024	*Photuris sp.*	F	Blount	TN	5.17.2012	19.3916	diurnal
ZHM025	*Photuris sp.*	F	Blount	TN	5.17.2012	15.6925	diurnal
ZHM026	*Photinus pyralis*	M	Knox	TN	5.22.2012	18.7807	nocturnal
ZHM027	*Photinus pyralis*	M	Knox	TN	5.22.2012	10.718	nocturnal
ZHM028	*Photinus pyralis*	M	Knox	TN	5.22.2012	12.281	nocturnal
ZHM029	*Photinus pyralis*	M	Knox	TN	5.22.2012	14.3121	nocturnal
ZHM030	*Photinus pyralis*	M	Knox	TN	5.22.2012	11.9154	nocturnal

Table 3.8: Continued

ID	Species	Sex	County	State	Date Coll.	Dry Wt.	Nocturnal/Diurnal
ZHM031	*Photinus pyralis*	M	Knox	TN	5.22.2012	18.6329	nocturnal
ZHM032	*Photinus pyralis*	M	Knox	TN	5.22.2012	11.2786	nocturnal
ZHM033	*Photinus pyralis*	M	Knox	TN	5.22.2012	16.123	nocturnal
ZHM034	*Photuris sp.*	M	Knox	TN	5.22.2012	12.1378	diurnal
ZHM035	*Photinus pyralis*	M	Knox	TN	5.22.2012	25.1928	nocturnal
ZHM036	*Photinus pyralis*	M	Knox	TN	5.22.2012	21.8312	nocturnal
ZHM037	*Photinus pyralis*	M	Knox	TN	5.22.2012	20.1542	nocturnal
ZHM038	*Photinus pyralis*	M	Knox	TN	5.22.2012	23.0217	nocturnal
ZHM040	*Photinus pyralis*	M	Knox	TN	5.22.2012	13.1818	nocturnal
ZHM049	*Photinus pyralis*	M	Anderson	TN	5.24.2012	18.8337	nocturnal
ZHM050	*Photinus pyralis*	M	Anderson	TN	5.24.2012	21.6196	nocturnal
ZHM051	*Photinus pyralis*	M	Anderson	TN	5.24.2012	14.3146	nocturnal
ZHM052	*Photinus pyralis*	M	Anderson	TN	5.24.2012	17.0291	nocturnal
ZHM053	*Photinus pyralis*	M	Anderson	TN	5.24.2012	15.0568	nocturnal
ZHM054	*Photinus pyralis*	M	Anderson	TN	5.24.2012	17.301	nocturnal
ZHM055	*Photinus pyralis*	M	Anderson	TN	5.24.2012	12.466	nocturnal
ZHM056	*Photuris sp.*	F	Anderson	TN	5.24.2012	14.3415	diurnal
ZHM057	*Photinus pyralis*	F	Anderson	TN	5.24.2012	15.8212	nocturnal
ZHM058	*Photinus pyralis*	M	Anderson	TN	5.24.2012	15.0777	nocturnal
ZHM059	*Photinus pyralis*	M	Anderson	TN	5.24.2012	16.9167	nocturnal
ZHM060	*Photinus pyralis*	M	Anderson	TN	5.24.2012	21.0189	nocturnal
ZHM061	*Photuris sp.*	F	Anderson	TN	5.24.2012	26.2302	diurnal
ZHM062	*Photinus pyralis*	M	Anderson	TN	5.24.2012	20.749	nocturnal
ZHM063	*Photinus pyralis*	M	Anderson	TN	5.24.2012	19.0741	nocturnal
ZHM064	*Photinus pyralis*	M	Anderson	TN	5.24.2012	22.6064	nocturnal
ZHM065	*Photinus pyralis*	M	Anderson	TN	5.24.2012	21.1092	nocturnal
ZHM066	*Photinus pyralis*	M	Anderson	TN	5.24.2012	14.0721	nocturnal
ZHM067	*Photinus pyralis*	M	Anderson	TN	5.24.2012	11.3409	nocturnal
ZHM068	*Photinus pyralis*	M	Anderson	TN	5.24.2012	31.2819	nocturnal
ZHM069	*Photinus pyralis*	M	Anderson	TN	5.24.2012	10.2211	nocturnal
ZHM070	*Photinus pyralis*	M	Anderson	TN	5.24.2012	10.4156	nocturnal

Table 3.8: Continued

ID	Species	Sex	County	State	Date Coll.	Dry Wt.	Nocturnal/Diurnal
ZHM071	*Photinus pyralis*	F	Anderson	TN	5.24.2012	55.6773	nocturnal
ZHM072	*Photinus pyralis*	M	Anderson	TN	5.24.2012	15.312	nocturnal
ZHM073	*Photinus pyralis*	M	Anderson	TN	5.24.2012	13.1093	nocturnal
ZHM074	*Photinus pyralis*	M	Anderson	TN	5.24.2012	19.9723	nocturnal
ZHM075	*Photinus pyralis*	M	Anderson	TN	5.24.2012	22.618	nocturnal
ZHM076	*Photinus pyralis*	M	Anderson	TN	5.24.2012	16.1429	nocturnal
ZHM077	*Photinus pyralis*	M	Anderson	TN	5.24.2012	15.3052	nocturnal
ZHM078	*Photinus pyralis*	M	Anderson	TN	5.24.2012	15.9745	nocturnal
ZHM079	*Photuris sp.*	M	Anderson	TN	5.24.2012	10.6745	diurnal
ZHM080	*Photinus pyralis*	M	Blount	TN	5.29.2012	12.4306	nocturnal
ZHM081	*Photinus pyralis*	M	Blount	TN	5.29.2012	11.2233	nocturnal
ZHM082	*Photinus pyralis*	M	Blount	TN	5.29.2012	15.3479	nocturnal
ZHM083	*Photinus pyralis*	M	Blount	TN	5.29.2012	13.7768	nocturnal
ZHM084	*Photinus pyralis*	F	Blount	TN	5.29.2012	36.9243	nocturnal
ZHM085	*Photinus pyralis*	M	Blount	TN	5.29.2012	6.5471	nocturnal
ZHM086	*Photinus pyralis*	M	Blount	TN	5.29.2012	6.9032	nocturnal
ZHM088	*Photuris sp.*	M	Blount	TN	5.29.2012	8.0635	diurnal
ZHM089	*Photuris sp.*	M	Blount	TN	5.29.2012	11.8835	diurnal
ZHM090	*Photuris sp.*	M	Blount	TN	5.29.2012	8.8611	diurnal
ZHM091	*Photuris sp.*	F	Blount	TN	5.29.2012	15.6231	diurnal
ZHM092	*Photuris sp.*	M	Blount	TN	5.29.2012	3.7788	diurnal
ZHM093	*Photuris sp.*	F	Blount	TN	5.29.2012	15.1603	diurnal
ZHM094	*Photuris sp.*	M	Blount	TN	5.29.2012	6.1867	diurnal
ZHM095	*Photuris sp.*	F	Blount	TN	5.29.2012	16.3924	diurnal
ZHM096	*Photuris sp.*	F	Blount	TN	5.29.2012	34.9143	diurnal
ZHM098	*Photuris sp.*	M	Butler	PA	5.31.2012	10.6714	diurnal
ZHM099	*Photuris sp.*	M	Butler	PA	5.31.2012	12.5835	diurnal
ZHM100	*Photuris sp.*	M	Butler	PA	5.31.2012	10.9934	diurnal
ZHM101	*Photuris sp.*	M	Butler	PA	5.31.2012	11.9693	diurnal
ZHM102	*Photinus pyralis*	M	Knox	TN	6.4.2012	18.2463	nocturnal
ZHM103	*Photuris sp.*	M	Knox	TN	6.4.2012	10.0387	diurnal

Table 3.8: Continued

ID	Species	Sex	County	State	Date Coll.	Dry Wt.	Nocturnal/Diurnal
ZHM104	*Photuris sp.*	M	Knox	TN	6.4.2012	10.2359	diurnal
ZHM105	*Photuris sp.*	F	Knox	TN	6.4.2012	27.743	diurnal
ZHM106	*Photinus pyralis*	M	Knox	TN	6.4.2012	11.7868	nocturnal
ZHM107	*Photinus pyralis*	M	Knox	TN	6.4.2012	20.3127	nocturnal
ZHM108	*Photuris sp.*	F	Knox	TN	6.4.2012	16.1843	diurnal
ZHM109	*Photuris sp.*	M	Knox	TN	6.4.2012	9.0399	diurnal
ZHM111	*Photuris sp.*	M	Knox	TN	6.4.2012	9.9843	diurnal
ZHM112	*Photuris sp.*	M	Knox	TN	6.4.2012	9.7681	diurnal
ZHM113	*Photuris sp.*	M	Knox	TN	6.4.2012	8.7332	diurnal
ZHM114	*Photinus pyralis*	M	Knox	TN	6.4.2012	17.1359	nocturnal
ZHM115	*Photinus pyralis*	M	Knox	TN	6.4.2012	23.7243	nocturnal
ZHM116	*Photuris sp.*	M	Knox	TN	6.4.2012	9.5415	diurnal
ZHM117	*Photinus pyralis*	M	Knox	TN	6.4.2012	9.5885	nocturnal
ZHM118	*Photinus pyralis*	M	Knox	TN	6.4.2012	18.2018	nocturnal
ZHM119	*Photinus pyralis*	M	Knox	TN	6.4.2012	16.4396	nocturnal
ZHM120	*Photinus pyralis*	M	Knox	TN	6.4.2012	14.1426	nocturnal
ZHM121	*Photinus pyralis*	M	Knox	TN	6.4.2012	20.8029	nocturnal
ZHM122	*Photinus pyralis*	M	Knox	TN	6.4.2012	14.9865	nocturnal
ZHM123	*Photinus pyralis*	M	Knox	TN	6.4.2012	16.4359	nocturnal
ZHM124	*Photinus pyralis*	M	Knox	TN	6.4.2012	15.8647	nocturnal
ZHM125	*Photinus pyralis*	M	Knox	TN	6.7.2012	17.1847	nocturnal
ZHM126	*Photinus pyralis*	M	Knox	TN	6.7.2012	25.9074	nocturnal
ZHM127	*Photinus pyralis*	M	Knox	TN	6.7.2012	14.6953	nocturnal
ZHM128	*Photinus pyralis*	M	Knox	TN	6.7.2012	14.6474	nocturnal
ZHM129	*Photinus pyralis*	M	Knox	TN	6.7.2012	19.0183	nocturnal
ZHM130	*Photinus pyralis*	M	Knox	TN	6.7.2012	20.7424	nocturnal
ZHM131	*Photinus pyralis*	M	Knox	TN	6.7.2012	18.6975	nocturnal
ZHM132	*Photinus pyralis*	M	Knox	TN	6.7.2012	14.2161	nocturnal
ZHM133	*Photinus pyralis*	M	Knox	TN	6.7.2012	20.4955	nocturnal
ZHM134	*Photinus pyralis*	M	Knox	TN	6.7.2012	10.5601	nocturnal
ZHM135	*Photinus pyralis*	M	Knox	TN	6.7.2012	11.5351	nocturnal

Table 3.8: Continued

ID	Species	Sex	County	State	Date Coll.	Dry Wt.	Nocturnal/Diurnal
ZHM136	*Photuris sp.*	F	Knox	TN	6.7.2012	17.0442	diurnal
ZHM137	*Photuris sp.*	M	Knox	TN	6.7.2012	9.0505	diurnal
ZHM138	*Photuris sp.*	M	Knox	TN	6.7.2012	10.2855	diurnal
ZHM139	*Photuris sp.*	F	Knox	TN	6.7.2012	17.1654	diurnal
ZHM140	*Photuris sp.*	M	Knox	TN	6.7.2012	9.2308	diurnal
ZHM141	*Photuris sp.*	M	Knox	TN	6.7.2012	10.6342	diurnal
ZHM142	*Photuris sp.*	M	Knox	TN	6.7.2012	11.1892	diurnal
ZHM143	*Photuris sp.*	M	Knox	TN	6.7.2012	10.9833	diurnal
ZHM144	*Photuris sp.*	M	Knox	TN	6.7.2012	21.5005	diurnal
ZHM145	*Photuris sp.*	F	Knox	TN	6.7.2012	21.0306	diurnal
ZHM146	*Photuris sp.*	M	Knox	TN	6.7.2012	11.3539	diurnal
ZHM147	*Photuris sp.*	M	Knox	TN	6.7.2012	11.2954	diurnal
ZHM148	*Photuris sp.*	M	Knox	TN	6.7.2012	9.1784	diurnal
ZHM149	*Photuris sp.*	F	Knox	TN	6.4.2012	24.2109	diurnal
ZHM150	*Photinus pyralis*	M	Knox	TN	6.4.2012	13.9819	nocturnal
ZHM151	*Lamprohiza splendidula*	M	Wynegem	Belgium		3.7129	nocturnal
ZHM152	*Lamprohiza splendidula*	M	Wynegem	Belgium		2.7754	nocturnal
ZHM153	*Lamprohiza splendidula*	M	Wynegem	Belgium		5.5188	nocturnal
ZHM154	*Lamprohiza splendidula*	M	Wynegem	Belgium		3.0523	nocturnal
ZHM155	*Lamprohiza splendidula*	M	Wynegem	Belgium		2.6771	nocturnal
ZHM156	*Lamprohiza splendidula*	M	Wynegem	Belgium		2.9798	nocturnal
ZHM157	*Lamprohiza splendidula*	M	Wynegem	Belgium		1.9954	nocturnal
ZHM158	*Lamprohiza splendidula*	F	Wynegem	Belgium		15.9232	nocturnal
ZHM159	*Lamprohiza splendidula*	F	Wynegem	Belgium		9.5511	nocturnal
ZHM160	*Lamprohiza splendidula*	F	Wynegem	Belgium		2.8909	nocturnal
ZHM161	*Lampyris noctiluca*	F	Wynegem	Belgium	7.22.2012	20.9336	nocturnal
ZHM162	*Lampyris noctiluca*	F	Wynegem	Belgium	7.22.2012	30.1196	nocturnal
ZHM163	*Lampyris noctiluca*	M	Wynegem	Belgium	7.22.2012	6.8737	nocturnal

CONCLUSION

Orbitrap mass spectrometers have become some of the most useful instruments for probing biological systems because of their ability to perform discovery-based analysis using a wide variety of analytical approaches. This thesis has summarized three investigations into small molecules effects on species and diseases. Three classes of biologically significant small molecules were analyzed using semi-targeted mass spectrometric methods.

A lipidomics method was developed to analyze the phospholipidome of *Candida Albicans* to investigate virulence. The losses of phosphatidylserine and phosphatidylethanolamine synthesis pathways affect the phospholipid composition of *C. albicans*. The enzymatic mutations studied caused changes in the amounts of phosphatidic acid, phosphatidylglycerol, phosphatidylinositol and phosphatidylserine while few changes were seen in phosphatidylethanolamine and phosphatidylcholine. Based on these results, *C. albicans* can balance the loss of these synthesis pathways using the Kennedy pathway as an alternate method.

Flux analysis was performed on coral reef samples to investigate black band disease effects on infection progression. Also, an R package was developed to improve analysis of isotope labelling experiments for this as well as future studies. The flux data show differences in metabolic activity and rates depending on where samples were taken with infected coral showing higher flux rates than healthy coral.

Finally, an HPLC/PDA/MS method was developed to examine the composition of lucibufagins (LBGs) that fireflies use as a defense against predation. Studies have shown a large variety in amounts and in the composition of LBGs that different firefly species use for defense. LBGs have a UV absorption at 300nm which made detecting potential unknown LBGs possible using a photodiode array detector in-line with the Orbitrap mass spectrometer. Statistical comparisons of these unknown masses show differences based on locations, species, and whether the species is diurnal or nocturnal.

This method can be applied to other studies on unknown metabolites that may be contributing to differences between species or disease states.

VITA

Abigail T. Farmer was born and raised in Chattanooga, TN. She completed her B.S. degree in Chemistry at Meredith College in Raleigh, NC in 2012. Currently she is a graduate student working in the laboratory of Dr. Shawn Campagna at the University of Tennessee, Knoxville.