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**Bioluminescência fúngica:
papel ecológico,
purificação e clonagem de enzimas**

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Universidade de São Paulo para obtenção do
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HANS EUGENE WALDENMAIER

Tese de Doutorado submetida ao Instituto de Química da Universidade de São Paulo como parte dos requisitos necessários à obtenção do grau de Doutor em Ciências - Área: Bioquímica.

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Resumo:

Waldenmaier, HE. **Bioluminescência fúngica: papel ecológico, purificação e clonagem de enzimas**. Tese de Doutorado - Programa de Pós-Graduação em bioquímica. Instituto de Química, Universidade de São Paulo, São Paulo.

Esta tese de doutorado descreve os estudos realizados para elucidar a biologia molecular da bioluminescência fúngica e sua relevância ecológica na natureza. A recente descoberta de que a luciferina fúngica é a 3-hidroxihiispidina permitiu a caracterização do metabolismo secundário da fenilalanina nos genomas recém-sequenciados e transcriptomas de micélios das espécies luminescentes *Panellus stipticus* e *Neonothopanus gardneri*. Adicionalmente os genomas e transcriptomas de variedades não luminescente de *P. stipticus* e *Lentinula edodes* serviram como respectivos controles. Em geral, os genes envolvidos no metabolismo secundário da fenilalanina em amostras luminescentes tinham expressão igual ou superior àquela de espécies não luminescentes. Um agrupamento de genes relacionados com a biossíntese de fenilalanina foi encontrado em ambos os genomas luminescentes e não luminescentes de *P. stipticus*. A abundância de genes transcritos neste agrupamento foi semelhante para as espécies luminescentes e não luminescentes de *P. stipticus*, mas a poliketídeo sintase tipo I em *P. stipticus* não luminescentes foi significativamente sub-regulada. Não foi encontrado agrupamento semelhante nos genomas de *N. gardneri* e *L. edodes*, sendo que os correspondentes homólogos estavam espalhados em diferentes loci.

Extratos de fungos podem ser preparados *in vitro*, com a adição de 3-hidroxihiispidina para produzir luz verde em abundância. A preparação de extratos proteicos de luciferase foi melhorada e a estrutura da luciferase, parcialmente purificada, foi investigada por espectrometria de massas. A presença de luciferase nos géis de purificação foi revelada usando-se luciferina e molécula similares à luciferina advindas de extratos de plantas.

O nicho ecológico nas vizinhas de cogumelos bioluminescentes foi investigado de duas maneiras, armadilhas adesivas com cogumelos artificiais de acrílico, iluminados com luz LED verde e através da observação direta de cogumelos bioluminescentes com fotografia no infravermelho com lapso de tempo. Os estudos ecológicos foram conduzidos nos biomas da Mata Atlântica e da Mata dos Cocais, no Brasil. Baratas, aranhas, tesourinhas, grilo e vagalumes tec-tecs foram os animais mais comuns que interagiram com os cogumelos. Todos estes animais podem agir como dispersores de propágulos e, em alguns casos, como defensores dos cogumelos.

Palavras-chave: bioluminescência fúngica, metabolismo secundário da fenilalanina, luciferase, ecologia

ABSTRACT

Waldenmaier, HE. **Fungal bioluminescence: ecological role, purification and cloning of enzymes.** Tese de Doutorado - Programa de Pós-Graduação em bioquímica. Instituto de Química, Universidade de São Paulo, São Paulo.

This PhD thesis describes the studies performed to elucidate the molecular biology of fungal bioluminescence and the ecological significance of the trait in the wild. The recent discovery that the fungal luciferin is 3-hydroxyhispidin has allowed for the characterization of phenylalanine secondary metabolism in the newly sequenced genomes and mycelium transcriptomes of luminescent *Panellus stipticus* and *Neonothopanus gardneri*, additionally the genomes and transcriptomes of a non-luminescent variety of *P. stipticus* and *Lentinula edodes* served as respective controls. In general the genes involved in phenylalanine secondary metabolism had greater or equal expression in luminescent samples than non luminescent. A cluster of genes related to the secondary metabolism of phenylalanine was found in both luminescent and non luminescent *P. stipticus* genomes. Transcript abundance of genes in this cluster was similar in both luminescent and non-luminescent *Panellus stipticus*, but the type I polyketide synthase in non luminescent *Panellus stipticus* was significantly down regulated. A similar gene cluster in the *N. gardneri* and *L. edodes* genomes was absent with corresponding homologues scattered at different genomic loci.

Cell free fungal extracts can be combined *in vitro* with the addition of 3-hydroxyhispidin to produce abundant green light. Preparation of proteinaceous luciferase extracts was improved and partially purified luciferase samples were investigated by mass spectrometry. The presence of luciferase in the separation gel was also evidenced by using luciferin and luciferin-like molecules from plant extracts.

The ecological niche surrounding bioluminescent mushrooms was investigated through two main means, glue traps with acrylic mushroom facsimiles that were internally illuminated with green LED lights and direct observation of bioluminescent mushrooms with infrared time lapse photography. Ecological studies were performed in the Atlantic rainforest (Mata Atlântica) and transitional Coconut Palm forest (Mata dos Cocais) biomes of Brazil. Cockroaches, spiders, earwigs, crickets, and luminescent click beetles were the most common animal interacting with mushrooms. All of these animals may be acting as fungal propagule dispersers and in some cases defense of the mushroom.

Keywords: fungal bioluminescence, phenylalanine secondary metabolism, luciferase, ecology

Lista de Abreviaturas e Siglas

Enzymes

PAL phenylalanine ammonia lyase
C4H trans-cinnamate 4-monooxygenase, cinnamate hydroxylase
C3H 4-coumarate-3-Hydroxylase
4CL 4-coumaryl:CoA-ligase

PKS Polyketide synthase (three types: I,II,III)
CHS Chalcone synthase a type III PKS in plants with 3 malonyl-CoA additions
SPS Styrylpyrone synthase a type III PKS in plants with 2 malonyl-CoA additions

HMG Hydroxymethylglutaryl-CoA synthase

Sequence Data Set Abbreviations

BL *Panellus stipticus*, Blount Co. Tenn USA - Bioluminescent
TU *Panellus stipticus*, Turkey
NG *Neonothopanus gardneri*, Altos PI BR - Bioluminescent
LE *Lentinula edodes*, Supermarket

BTNL The collective abbreviation for all four (BL, TU, NG, LE) datasets.

PI An additional transcriptome subset of NG from a preliminary sequencing effort.
Each PI transcript has expression fold change ratio between luminescent and non-luminescent mycelium

DNA: “_scaffold_” ex. BL_scaffold_4, BL_scaffold_23
Long sub-chromosome length of assembled DNA sequence.

Genes: “_mg_” ex. BL_mg_002894, NG_mg_013453
MAKER predicted gene sequence with intron/exons, CDS, translation and scaffold location.

Transcript: ex. LE012345, TU006589
Bowtie assembled transcript has associated replicate transcript abundance values.

IPR - Interproscan annotation ex. IPR005922 - phenylalanine ammonia-lyase

GO - Gene Ontology annotation ex. GO:0003824 - Catalytic activity

KEGG - Kyoto Encyclopedia of Genes and Genomes annotation:
ex. KEGG:ko00360 Phenylalanine metabolism

Table of contents

1. Introduction	12
1.1. Historic fungal bioluminescence observations	12
1.2. Modern history	16
1.3. The molecular nature of bioluminescence	21
1.3.1. Bioluminescent bacteria	22
1.3.2. Fireflies	23
1.3.3. Luminescent dinoflagellates	24
1.3.4. Cypridina	26
1.3.5. Coelenterazine based bioluminescent systems	27
1.3.6. Other terrestrial bioluminescence	30
1.4. The current biology of fungal bioluminescence	31
1.5. The metabolic pathway system of bioluminescence in fungi	34
1.5.1. Phenylalanine ammonia lyase	35
1.5.2. Cinnamate 4-hydroxylase	35
1.5.3. Coumaroyl 3-hydroxylase	36
1.5.4. 4-coumaryl:CoA-ligase	37
1.5.5. Polyketide synthase	38
1.6. Related chemistries	40
1.7. Function and ecology of bioluminescence	41
2. Objectives	45
3. Methods	45
3.1 Field studies	45
3.1.1. Locations and fungi	45
3.1.2. Encountering bioluminescent mushrooms	48
3.1.3. Glue trap arthropod collection and analysis	49
3.1.4. Infrared monitoring and analysis	50
3.2. Laboratory studies	51
3.2.1. Mycelium cultures	51
3.2.2. Luciferase enzyme extraction	53
3.2.3. Luciferin extraction methods	54
3.2.4. In vitro bioluminescence assay	55
3.2.5. DNA extraction from mycelium	55
3.2.6. RNA extraction from mycelium	58
3.2.7. Genome sequencing	59
3.2.8. Transcriptome sequencing	60
3.2.9. Proteomics	60
3.2.10. Bioinformatics	61
4. Results	62
4.1. Ecology	62
4.1.1. Bioluminescent mushrooms in the forest	62
4.1.2. Arthropods captured in glowing mushroom facsimiles	66
4.1.3. Arthropod activity on and around luminous mushrooms	72

4.1.3.1. IR infrared Atlantic rainforest	72
4.1.3.2. IR infrared transitional palm forest	75
4.2. Enzymology	80
4.2.1 Comparison of light intensity of luminous cultures	81
4.2.2. Experiments with the crude extraction of luciferase from <i>N. gardneri</i>	82
4.2.3. Luciferase partial purification	85
4.2.4. Equisetumpyrone and fungal β -glucosidase	87
4.2.5. DNA extraction	89
4.2.6. RNA extraction	93
4.2.7. Genome and transcriptome sequencing and raw data analysis	93
4.2.8. In silico dissection of hispidin metabolism	96
4.2.8.1. Phenylalanine ammonia lyase	97
4.2.8.2. C4H and other cytochrome p450s	99
4.2.8.3. 4-coumaryl:CoA-ligase	104
4.2.8.4. Hispidin synthase: polyketide synthases	105
4.2.8.5. Signs of fungal luciferase	108
5. Discussion	116
5.1. The molecular nature of fungal luminescence	116
5.1.1. The panellus phenylalanine secondary metabolism gene cluster	116
5.1.2. Clustering of other secondary metabolism genes	119
5.1.3. Cytochrome p450 genes	119
5.1.4. Genomes and Transcriptomes	120
5.1.5. Luciferase substrates	123
5.1.6. The regulation of fungal bioluminescence	126
5.2. Ecology discussion	126
5.2.1 The ecological significance of fungal bioluminescence	126
5.2.2 Nocturnal observations of insects and bioluminescent mushrooms	129
6.0. Conclusions	132
7.0. References	133
Appendix list	144

1. Introduction

The human eye is often caught by objects having high contrast with their surrounding, this text for example. An organism's ability to see and to be seen involve it in a complex duel with its environment for survival. Bioluminescent fungi like other bioluminescent organisms are a spectacular natural example of an object having high visual contrast with its surrounding environment in darkness. Aside from luminous insects, fungi are the most commonly observed bioluminescent organisms outside of the ocean. Luminescent fungi are found in two forms in the wild, luminescing mushroom fruiting bodies and mycelium infested decaying wood known as foxfire (saporema em Portuguese). This thesis starts with a pre-1900 historic review of Edmund Harvey's: A History of Luminescence (1957) focused on the history of fungal bioluminescence knowledge.

1.1 Historic Fungal Bioluminescence Observations

While ancient human civilizations undoubtedly were aware of bioluminescent fungi since glowing mushrooms and ground mycelium are easily seen in total darkness (NO LIGHTS!) as they were aware of other luminescing creatures, older ancient texts from around the world are devoid of any mention of luminous fungi aside from some vague reference to luminous trees by ancient asian cultures which could relate to glowing fungal mycelium. Aristotle and Pliny the Elder were the first to specifically write of glowing mushrooms. Aristotle remarked about how the eerie glow of mushrooms can be seen at night but not in the day in a general discussion of "fiery and glittering" things. Pliny the Elder noted the presence of the glowing mushrooms in tops of olive trees (likely the species *Omphalotus olearius*) and their use for antidotes and confections, and that one must know how and where to find them in order to encounter them. Following these mentions by Greek and Roman writers there was little to no mention of fungal bioluminescence during the dark ages of Europe whose culture was focused on things aside from the natural world. Not until the scientific renaissance of the fifteenth and sixteenth centuries

did accounts of fungal bioluminescence begin to accumulate. Gonzalo Fernandez de Oviedo, a Spanish chronicler of the the new world in Panama and the West Indies wrote the first important record of the natural history of the new world, in which he noted several examples of bioluminescence including foxfire. He noted that natives and europeans used foxfire to help aide groups moving through the forest at night, with the head of the line placing a large piece of foxfire on their back giving a visual que to others behind as to the direction of the group. Glowing wood was not new to Oviedo, he was aware of Aristotle's writings and mentioned that he had witnessed glowing wood previously in Spain. Further he commented that the glowing wood could be valuable if the glow persisted for more than just one or two days. Additional writings about glowing wood occurred throughout the fifteenth and sixteenth centuries, namely by a nordic priest Olaus Magnus who mentioned the use of rotting wood to light paths at night, and the first book on luminescence was authored by Conrad Gesner in 1555 which mentioned glowing wood. Gesner was critical of the previous reports of glowing live plants, suggesting that their luminosity was attributed to the reflection of the stars and moon. Prior to the seventeenth century, writings of luminous fungi and glowing wood were solely focused on documenting accounts and early efforts to name and categorize species of glowing mushrooms. In the seventeenth century the focus began to change from mere observation of the occurrence to trying to understand the nature of the light emission itself.

The first real scientific observations of fungal bioluminescence were of foxfire by Francis Bacon in his book *Sylva Sylvarum* (Bacon 1627), where he noted that the light is variable in the wood, specific tree species whose wood often glows, that the roots of dead trees often glowed more strongly than trunk wood, luminescence spreads from glowing parts to dark parts over time, one can make more luminous wood by placing non-glowing wood on top of glowing wood, cold weather does not seem to greatly inhibit already glowing wood. Most of Bacon's

observations are still viewed as accurate today. The first true experiments trying to understand the nature of glowing wood were performed by Robert Boyle (Boyle 1667). Boyle placed a piece of glowing wood in a vacuum, he pumped the air out slowly and slowly the luminosity of the piece of wood diminished as the vacuum got stronger, and then once the vacuum was broken the light quickly resumed. Boyle then compared the properties of the glowing wood to that of a burning coal which included: both are luminaries - not reflectors, both need air to glow, both can be quenched by water, and that cold air does not quench either. He also noted the differences between the two, while one can stomp out a burning coal, glowing wood cannot be quenched in the same way. Glowing wood will resume glowing after a prolonged vacuum a coal will not, coal gives off smoke, glowing wood does not. Finally, as a coal burns it gets smaller, the same cannot be said of glowing wood. Prior to the microscopic observations by Henry Baker in 1742 and Baron von Meidinger in 1777 it was thought that the glow of wood was due to some inherent property of the wood, following the microscopic observations it was becoming clearer that the glow was due to some form of "Animalcules" that fed upon the wood. Throughout the later part of the eighteenth century, as gas separation technology developed, several experiments were developed to investigate the effect of these gases on luminous wood. Frederic Achard found that luminescence persisted under partial vacuum, in pure oxygen, but in nitrous air the light disappeared quickly and in carbon-dioxide rich air the light disappeared after about an hour. Achard also argued that luminous wood was not due to fluorescence or phosphorescence since wood dried in the dark and then without exposure to light was rehydrated regained light emission. Von Humboldt in 1799 continued these gaseous studies and further confirmed that oxygen was required and that carbon dioxide and nitrogen gas inhibited light emission, he further showed that even in the presence of oxygen light could be inhibited by high temperatures, acids and alcohols. While von Humboldt's observations were true his

conclusion that the light was a result of an equilibrium among oxygen, carbon dioxide, water and the organic matter of the wood was overall wrong.

Along the same line of gaseous experiments C. W. Boeckmann focused specifically on the comparison of properties of luminous wood and phosphorus luminescence, as one of the main hypothesis at the time was that luminous wood was a result of the oxidation of elemental phosphorus. He concluded that while there are some similarities between the two, the differences in luminescence properties were strong enough to exclude phosphorous luminescence as the mechanism of action in rotting wood. Placidus Heinrich in 1815 found that when luminous wood was placed in a closed container the amount of gas did not change much over time, suggesting the consumption of gases (oxygen) was in step with the production of gaseous byproducts (carbon dioxide).

It was not until the 1800s that the connection between glowing wood and fungi began to be made. The French Chemist, de Fourcroy noted that when wood started luminescing it also developed the odor of mushrooms. Esenbeck, Noggerath, and Bischoff in 1823 definitively described the source of light from luminous roots (rhizomorphs) to be from some living organism, and that the light emission was a part of a living biological process and not from the decomposition of the wood in itself. Johann Heller in 1853 had definitive proof that luminous wood was the result of fungi through microscopic observation that the light originated from threads of mycelium and through subsequent isolation of the mycelium from the wood which continued to grow as long as it was in vegetative growth and not after death. Finally, in 1855 R. Harting proved that the growths on luminescent roots (rhizomorphs) were the mycelium of the common honey mushroom, *Armillaria mellea*. Throughout the nineteenth century most of the focus of bioluminescent fungus researchers was identifying new species and starting cultures,

the pioneer of this field was F. Ludwig and following him in the early twentieth century was by Hans Molisch.

1.2 Modern history

The major foci of twentieth century fungal bioluminescence research continue to this day, namely the continued effort to identify new species, understanding the ecological and evolutionary significance of the trait, and work to understand the molecular mechanism of light emission in fungi.

The commonly found northern hemisphere species *Panellus stipticus* was noted for its luminosity when found in eastern North America, but other strains of this species from around the world lacked light emission (Macrae 1937, Airth and Foerster 1964, Jin et al. 1991). In the early 20th century mating and intercompatibility studies were performed between the different geographic isolates (Macrae 1937), crosses between luminous and nonluminous strains of *P. stipticus* resulted in all F1 offspring being luminous suggesting that the bioluminescence trait is governed by a single dominant allele in *P. stipticus*. Inheritance studies were performed in the 1990s by Linge et al. (1992) again with *P. stipticus*. In these experiments luminous monokaryotic isolates of *panellus* were mated with naturally occurring mutant non-luminous monokaryotic mycelium isolated from weakly glowing mushrooms growing in a larger cluster of brighter mushrooms. They found that when two luminous monokaryon strains were crossed the resulting dikaryotic mycelium was most luminous, when a luminous strain was crossed with a non-luminous strain light emission was midlevel but still observable with the naked dark-adapted eye. Crosses between the naturally occurring non-luminescent mutants resulted in a mix of luminous and nonluminous dikaryotic mycelium, suggesting that the non-luminous strains contained compatible genes for luminescence. While the work of Linge et al. was consistent with the earlier work of Macrae, the complementation patterns of the bioluminescence trait in

offspring of non-luminous monokaryons suggest the existence of at least three allelic loci involved in the fungal bioluminescent trait.

In the twentieth century some attention was given to the possible function of bioluminescence in fungi. It has been noted by many observers that arthropods and gastropods were found feeding on luminous mushrooms (references in Petersen and Bermudes 1992, Sivinski 1981). Further it was noted that some species of mushrooms luminesce most strongly from the spore containing gills of the mushroom pileus (Okane et al. 1990). This led some to suggest that bioluminescence is a means to attract spore dispersal vectors (McAlpine 1900, Ewart 1906, Johnson 1919, Lloyd 1974). At the same time, mycophagy by arthropods and gastropods of non-luminescent mushrooms is a common occurrence, and it was noted that the non-luminescent european varieties of *P. stipticus* were also eaten by slugs (Johnson 1919). Others have doubted the idea that bioluminescence is a way of spore dispersal (Buller 1924, Watling 1981; O'Kane et al. 1990), suggesting that light emission is the result of some beneficial metabolic reaction and plays no significant role ecologically (Herring 1994). It was not until 1981 when the first experiment directly testing the role of light in the ecological niche surrounding bioluminescent mushrooms at night were performed by Sivinski. Sivinski collected luminous mushrooms and placed them in sealed glass tubes covered in an insect trap glue, at the same time control traps were prepared by extinguishing luminescence by soaking in ethanol and placing in similar glue covered tubes. The glue traps were set out at night and collected the following morning and organisms trapped in the glue were identified and counted. Sivinski found that several orders of insects were found in statistically greater numbers on the luminescent glue traps, Collembola and Diptera. Several predatory groups were also found on luminous glue traps in numbers bordering on significance, spiders, ants and earwigs. This led Sivinski to propose several hypotheses for the function of bioluminescent light from fungi: (1) luminescence

may be a method of attracting spore dispersers, (2) attraction of carnivores of fungivores, (3) attraction of fertilizers, (4) repulsion of negatively phototropic fungivores, (5) attraction of fungivores of other fungal competitors, (6) and the light may be an aposematic warning signal of mushroom toxicity.

The chemical nature of fungal bioluminescence began to be unraveled in the twentieth century. In all other previously characterized bioluminescent systems (discussed next section), cellular extracts could be separated into two fractions that when combined and with additional factors can yield light *in vitro*. This is the so called *in vitro* hot-cold assay for bioluminescence, the hot fraction is the heat stable aqueous extract containing a small molecule that is commonly called the luciferin, the cold fraction is protein rich and contains the heat labile enzyme commonly termed the luciferase (Wilson and Hastings 1997). The hot-cold assay was first developed in the late 19th century by Dubois while studying bioluminescent clams and fireflies (Dubois 1885, Dubois 1887). Early attempts to extract these components from fungi and perform the *in vitro* hot and cold assay were unsuccessful (Harvey 1952). In the late 1950s, the first report of the successful cell free light emission from fungal extracts was reported by Airth and McElroy. Initially the Airth group prepared extracts from dried mycelial mats of *Collybia velutipes* and *Armillaria mellea*. The hot extract was prepared by boiling powderized mycelium in a phosphate buffer, cooling, and centrifuging to remove debris. The cold extract was prepared by grinding mycelium at cold temperatures in a phosphate buffer, centrifugation to remove debris, and then an ammonium sulfate precipitation of proteins to concentrate the luciferase, and a final resuspension in a small volume of buffer. When the cold and hot extract were combined with the addition of NAD(P)H, light was observed *in vitro*. In their initial report Airth and Foerster demonstrated that the luciferase was indeed heat labile and that the luciferin is quite unstable losing most of its activity in buffer within hours of extraction. Noting that the peak

wavelength of emission is 530 nm, they speculated the involvement of a flavin, but the addition of various flavins and other compounds in the presence and absence of the hot extract did not stimulate or enhance light emission. In a follow up publication, Airth and Foerster demonstrated that the cold extract can be separated into two fractions by ultracentrifugation, both proteinaceous fractions are required for light emission *in vitro* with fungal hot extract. They found that the order of addition of supernatant, pellet, NAD(P)H, and hot extract determined the kinetics of the reaction. When the hot extract and NAD(P)H were first incubated with the pellet and then addition of the supernatant, light emission increased steadily. Alternatively when the hot extract, NAD(P)H and supernatant were incubated first followed by addition of the pellet, the *in vitro* light emission increased rapidly and then plateaued. This suggested that the NAD(P)H dependent activity was associated with the supernatant and that the light emitting reaction was associated with the pellet, from these results they proposed a two step mechanism where first the luciferin is reduced by a NAD(P)H-dependent reductase followed by the oxidation of the reduced luciferin by the luciferase yielding light and the oxyluciferin. In 1964, Airth and Foerster turned their attention to *P. stipticus* and the nature of the lack of light emission from the European varieties and the ability to cross react extracts from different luminous species. When supernatant and pellet fractions of the European *P. stipticus* were cross reacted with fractions from *C. velutipes*, light emission was absent, while when fractions were cross reacted between luminescent *P. stipticus* and *C. velutipes* light emission was observed. Further, when fractions of non-luminescent *P. stipticus* were added to the complete reaction of *C. velutipes* light emission was not greatly inhibited. These results suggested that the mechanism of light emission is conserved among bioluminescent fungi and that the lack of light emission in the European varieties is due to a lack of protein expression and not due to an inhibitor.

Following the work done by Airth, Foerster and McElroy, work on the chemical nature of bioluminescence did not progress much for several decades with multiple groups attempting to repeat the enzymatic reaction without success. In the meantime, Osamu Shimomura was able to extract compounds from luminous fungi that when stimulated by the addition of H_2O_2 , iron(II), and superoxide anion resulted in light emission, without the need of a luciferase (Shimomura et al. 1993). Additionally, Shimomura demonstrated the involvement of superoxide dismutase with luminescent tissues and the production of these chemically induced luminescing compounds (Shimomura 1992, Shimomura 2006). These results led Shimomura to propose a mechanism of light emission which did not rely on enzymes suggesting that fungal bioluminescence is a form of spontaneous chemiluminescence.

The chemical nature of fungal bioluminescence was finally resolved in the early 21st century when the results of Airth and Foerster were confirmed by Oliveira and Stevani (2009). When the hot extract of dried *N. gardneri* mushrooms and the cold extract of *N. gardneri* mycelium were combined in vitro with NAD(P)H, light emission was observed. The *in vitro* light emission spectrum matched to the *in vivo* light, both had a maximum wavelength of emission of 533 nm. Moreover, Oliveira and Stevani were able to perform cross reactions between different species of mushrooms, *Geronema viridilucens* and *Mycena lucentipes*. With the confirmation that fungal luminescence is enzyme-catalyzed and a two step reaction, Oliveira and Stevani demonstrated that the mechanism of light emission is conserved among the four major groups of luminescent fungi (groups discussed in a later sections) by performing cross reactions of hot and cold extracts among lineages (Oliveira et al. 2012). The most recent development in the long history of fungal bioluminescence was the identification of the fungal luciferin by Purto et al. 2015, namely 3-hydroxyhispidin (Fig. 1F) reacts directly with the fungal insoluble pellet extract to produce light in the presence of oxygen, the precursor hispidin is hydroxylated by a

NAD(P)H-dependent hydroxylase found in the supernatant. Thus the results of Purto et al. suggest a different NAD(P)H dependent first step a hydroxylation of the luciferin precursor instead of a reduction prior to the luciferase reaction.

1.3 The molecular nature of bioluminescence

Bioluminescence is the enzyme catalyzed emission of visible light from a living organism (Harvey 1957). Spectra of light from organisms and the underlying chemistry is diverse ranging from violet to red light (Haddock and Case 1999, Herring and Cope 2005, Viviani et al. 1999). A luciferin is a small molecule that generates light upon a chemical reaction with oxygen catalyzed by an enzyme generically called luciferase (Wilson & Hastings 1998; Shimomura 2006). Other luciferins interact with photoproteins to produce light. One of the main differences between luciferases and photoproteins is the dependence of oxygen in cell-free luminescent reactions, photoproteins do not require oxygen for in vitro luminescence although oxygen is required for active photoprotein synthesis, the net luminescence is directly correlated with the protein amount, as the photoprotein acts as reactant in the reaction being consumed (Shimomura 2006). One can also consider photoproteins a subset of luciferases with low luciferin turnover, and where the luciferin binds covalently with the catalyzing protein. Luciferases require oxygen in the cell-free luminescence reaction and act as a catalyst (enzyme) which can be recycled. The total amount of light is dependent on the quantity of luciferin substrate. At the chemical level, most bioluminescent light is generated as a result of the decomposition of a four-membered ring dioxetanone (Wilson & Hastings 1998). These strained and energy-rich peroxides require low energy to break, but their decomposition yields a molecule in the electronically excited state, whose decay to the ground state is accompanied by light emission. Some bioluminescent systems do not employ a dioxetanone intermediate, but instead an acyclic peroxide (a hemiperoxyacetal), such as bacterial systems.

Figure 1. Discussed compounds a. Bacterial FMN:tetradecanal, b. Firefly luciferin, D-luciferin, c. Dinoflagellate luciferin, d. Cypridina luciferin e. Coelenterazine f. Fungal luciferin, 3-hydroxy hispidin. g. Equisetumpyrone. h. Hispolon, i. inoscavin A. Stars on luciferins indicate primary carbon atoms lost as carbon dioxide during the luciferase catalyzed reaction.

1.3.1. Bioluminescent bacteria

Luminescent bacteria are found in marine, freshwater and terrestrial environments. They are often found as free-living species in the ocean, growing on dead fish or meat, as a part of

the gut flora of some marine fish, and as parasites of arthropods (Wilson and Hastings 2013). Three genera contain most of the luminescent bacteria, *Aliivibrio*, *Photobacterium*, and *Xenorhabdus* with only the later found in terrestrial environments (Meighen 1991). Bacteria emit blue light at 490 nm from the decomposition of the hemiperoxyacetal formed by the reaction among reduced flavin mononucleotide (FMNH₂), oxygen and a long-chain aldehyde (Fig. 1A) inside the active site of the luciferase (Cormier & Strehler 1953). The genes for bacterial bioluminescence are organized into the bacterial *lux* operon (Engebrecht and Silverman 1984). While there is great diversity of *lux* operons among species, resulting in over 21 different *lux* genes, there are five common *lux* genes in all bioluminescent bacterial species (Meighen 1994). These include *luxA* and *luxB* which encode the bacterial luciferase, and genes for the fatty-acid luciferin reductase complex, *luxC*, *luxD* and *luxE* (Meighen 1991). Moreover, species-specific bioluminescence genes are also found in the *lux* operon (Meighen 1994). Depending on the species, several flavin-reductases supply reduced flavin to the luminescent reaction a common one being homologous to the *E. coli* flavin reductase Fre (Zenno & Saigo 1994). The reaction does not produce a dioxetanone intermediate, but instead a FMN hydroperoxide, which on its turn adds to a long-chain aldehyde producing a hemiperoxyacetal, whose decomposition via the so-called Chemically Initiated Electron Exchange Luminescence (CIEEL) leads to the formation of hydroxy-FMN in the electronically excited state (Eckstein et al. 1993; Villa & Willetts 1997; Stevani & Baader 1999; Berkel et al. 2006). The bacterial luciferase and the NAD(P)H-dependent flavin-reductase form a complex as demonstrated by affinity studies (Watanabe & Hastings 1982) and kinetic experiments (Tu 2007).

1.3.2. Fireflies

Fireflies, luminescent beetles, are the most commonly observed bioluminescence by humans, they are found on all continents except Antarctica. There are three families of fireflies

within the Arthropod order of Coleoptera, the Lampyridae which includes the common firefly, the Phengodidae which includes luminescent larvae called railroad worms, and the Elateridae which includes click beetles. Depending on firefly species, the in vivo light emission peaks range from 552 to 582 nm (Seliger & McElroy 1964). The firefly luciferase catalyses the reaction of firefly luciferin (Fig.1B) with ATP and molecular oxygen, resulting in light emission, CO₂, AMP and oxyluciferin. The mechanism involves the formation of a dioxetanone ring common to other bioluminescent systems (Shimomura et al. 1977). The mechanism of light emission in fireflies is one of the best characterized with the structures of firefly luciferin and firefly luciferase determined (White et al. 1961; Conti et al. 1996). All three clades of bioluminescent beetle share the same basic bioluminescent mechanism and catalyse the same luciferin as the well-studied lampyrids, although species of phengodids and elaterids have homologous luciferases that emit different colors (Wood et al. 1989; Viviani et al. 1999). cDNA clones of firefly luciferase have been isolated and translated in vitro (Wood et al. 1984). The firefly luciferase likely evolved from an AMP-CoA-ligase lacking or having low oxygenase activity following an increase in available D-luciferin, an orthologous luciferase like AMP-CoA-ligase from the larvae of the beetle family Tenebrionidae is capable of low and red light emission with D-luciferin (Viviani et al. 2010, Viviani et al. 2013).

1.3.3. Luminescent dinoflagellates

Dinoflagellates are the main protist that bioluminesce, and are responsible for the majority of bioluminescence in the surface ocean (Tett 1971, Valiadi and Iglesias-Rodriguez 2013). Many luminescent species also form blooms, called red-tide, causing bright bioluminescent displays along ocean shores and in bays at night. Light production in dinoflagellates occurs in organelles called scintillons which contain luciferin, luciferase and other proteins related to bioluminescence (Desa and Hastings 1968). Bioluminescence in

dinoflagellates occurs in photosynthetic and heterotrophic species, although majority are photosynthetic (Wilson and Hastings 1997). The gene responsible was cloned (Bae & Hastings 1994), and the crystal structure for functional 35 kDa dinoflagellate luciferase was solved by Schultz et al. (2005). In photosynthetic species the luciferase gene is present in multiple copies, the gene itself contains three homologous repeating domains each with high sequence similarity at the amino acid level (Li et al. 1997). In addition to the luciferase, many photosynthetic species of luminescent dinoflagellates contain a luciferin binding protein, which keeps luciferin at physiological pH until light is stimulated (Schmitter et al. 1976). Both the luciferase and luciferin binding protein share some sequence similarity in their N-terminal gene region (Li and Hastings 1998). In some photosynthetic species the transcription of the luciferin binding protein is very high accounting for 1% of the total transcriptome (Dunlap and Hastings 1981). In the heterotrophic species *Noctiluca scintillans*, the luciferase is quite different than photosynthetic species, the luciferase gene only contains a single domain that is homologous to the three domains of the photosynthetic luciferase (Liu and Hastings 2007). Additionally, a second domain of *N. scintillans* luciferase has high sequence similarity with the photosynthetic luciferin binding domain. The dinoflagellate luciferin (Fig. 1C) is a linear tetrapyrrole with similarity to chlorophyll a, the luciferin fluoresces in blue (475 nm) but the oxyluciferin is non-fluorescent in water (Nakamura et al. 1989). The luciferin structure has only been determined with certainty from one species, *P. lunula* (Valiadi and Iglesias-Rodriguez 2013), it is assumed that it is the same in other species as the luciferin and luciferase of different dinoflagellate species can cross react (Topalov and Kishi 2001). The bioluminescent pathway does not involve a dioxetanone intermediate (Stojanovic & Kishi 1994).

There are several levels of regulation of bioluminescence in dinoflagellates, firstly, light emission is under diurnal control, with light emission being much brighter at night than in the day

which is negligible (Valiadi and Iglesias-Rodrigues 2013). In *L. polyedrum*, the scintillions are destroyed each morning at dawn and are resynthesized at dusk (Steidinger and Tangen 1997). In *P. lunula*, the diurnal control is very different, scintillion count remains constant throughout the day-night cycle, but the location of the scintillions change, with them being located at the periphery of the cell at night and relocating via actin transport to the center of the cell during the day, this movement is opposite of that of chloroplasts which are on the cellular periphery during the day (Heimann et al. 2009). In addition to the molecular circadian control of luminescence, the bioluminescent system can be photoinhibited, with blue light being the most inhibitory, the mechanism of photoinhibition is not well understood (Esaias et al. 1973).

Additionally, the krill and dinoflagellate systems are quite similar. Both use a very similar tetrapyrrole compound as luciferin, differing only by a hydroxyl group moiety (Nakamura et al. 1988; Nakamura et al. 1989). In fact, the chemical pathways involved are the same, and luminescence was observed in a cross-reaction between krill luciferin and dinoflagellate luciferase, and vice-versa (Dunlap et al. 1980). The krill species *Euphausia pacifica* and *Meganyctiphanes norvegica* are the most studied bioluminescent Euphausiids, and both species are widely distributed in marine environments (Shimomura 2006).

1.3.4. Cypridina

Cypridina hilgendorffii, an ostracod crustacean has been used as a tool to understand the biochemistry of bioluminescence throughout the 20th century. When disturbed it secretes a luminous mucus (light in the range of 450–460 nm) from a specialized gland with two types of cells, one cell type secreting luciferin and the other secreting luciferase, resulting in a bright blue cloud of bioluminescence (Shimomura & Johnson 1970; Shimomura 2006). The structure of the luciferin (Fig. 1D) was determined by Kishi et al. (1966) and the luciferase was cloned by Thompson et al. (1989). Shimomura & Johnson (1971) demonstrated the involvement of a

dioxetanone intermediate in the luminescence reaction mechanism. The luciferase of *Pyrocypria noctiluca*, in the same class as *C. hilgendorffii*, has also been cloned, and the two homologous proteins have 83.1% sequence identity (Nakajima et al. 2004). Moreover, several coastal luminous fish also utilize Cypridina luciferin although their luciferases are likely analogous to *C. hilgendorffii* (Shimomura 2006).

1.3.5 Coelenterazine-based bioluminescent systems

Coelenterazine (Fig. 1E) is used as luciferin by many analogous systems, such as the protozoan *Thalassicollin* and six other animal phyla: Arthropoda, Cnidaria, Mollusca, Ctenophora, Chordates and Echinoderms (Campbell & Herring 1990; Shimomura 2006). All bioluminescent species that use coelenterazine are marine organisms. Notwithstanding the luciferin molecule is the same for these systems, the enzymes that catalyse the luminescent reaction are similar, but of independent origin. Only some species synthesize their own coelenterazine the rest relying on dietary sources, as there are many non-luminous species that contain large quantities of coelenterazine (Haddock et al. 2001). Coelenterazine can be used by the organism either with a luciferase or with a photoprotein.

Live specimens of *Aequorea victoria*, a hydrozoan medusa, emit green light (508-509 nm) from hundreds of photocytes found in a ring along the edge of the jellyfish's umbrella (Shimomura 2006). Luminescence of the cell-free pH 4.0 extract of the photocytes can be stimulated by the addition of a Ca(II) containing solution, the resulting light is a bright and short flash. Addition of coelenterazine, the *Aequorea* luciferin (Inoue et al. 1975), to the spent solution containing Ca(II) does not luminesce. In order to regain light emission, the Ca(II) and the oxyluciferin must first be removed from the protein containing solution and then incubated with fresh coelenterazine, following this incubation in the Ca(II) free solution a bright flash of light can be stimulated again by the addition of a Ca(II) solution (Shimomura 2006). This cycle of Ca(II)

stimulated light emission and regeneration in a Ca(II) free environment greatly differs from the traditional luciferin-luciferase reaction which is rather continuous as long as luciferin and other cofactors are present, given this differentiation the luciferases with this pattern are often referred to as photoproteins. The purified and stable substance which emits light when stimulated by Ca(II) was found to be a 21 kDa protein, termed aequorin, with coelenterazine-2-hydroperoxide stabilized in a hydrophobic pocket of the protein by hydrogen bonding to several amino acids including Try184 (Head et al. 2000). The binding of two Ca(II) ions to aequorin causes a conformational change causing the protein to open causing coelenterazine-2-hydroperoxide to decompose to coelenteramide and CO₂ with blue 460-465 nm light emission, the open protein is termed apo-aequorin. The green light observed in living specimens is due to green fluorescent protein, which emits green light through resonance energy transfer from aequorin and subsequent green fluorescence. For resonance energy transfer to occur the distance between GFP and aequorin must be less than 100 Å. Both *Aequorea* and GFP are found in high concentrations in the photocytes of *A. victoria* (Morise et al 1974). In addition to *Aequorea*, luminescence is common in the hydrozoans with the cDNA from *Obelia*, *Phialidium*, *Mitrocoma* expressed in *E. coli* to produce obelin, phialidin(clytin), mitrocomin respectively (Illarionov et al. 1995, Inouye and Tsuji 1993, Fagen et al. 1993). These additional hydrozoan photoproteins are similar to aequorin, all are stimulated by calcium, use coelenterazine as a substrate, and have the same size. These four proteins are structurally similar with high sequence conservation among them.

The sea pansy *Renilla reniformis*, an anthozoan, is another coelenterazine-based bioluminescent system. Like *Aequorea* the blue light of its luminescent reaction undergoes resonance energy transfer to an analogous GFP to emit green light in vivo, but unlike *Aequorea* its luciferase does not cycle between photoprotein and apo-photoprotein conformations

stimulated by Ca(II), the Renilla luciferase catalyzes coelenterazine as long as it is present. There are multiple levels of regulation of free coelenterazine available to the luciferase. Firstly, the majority of coelenterazine *in vivo* is in a sulfonated form (coelenterazine enol-sulfate) which is converted to free coelenterazine by luciferin-sulfokinase in the presence of 3',5'-diphosphoadenosine (Cormier et al. 1970). Secondly, like the hydrozoan luminescent systems, luminescence is stimulated by influx of Ca(II) that releases coelenterazine in Renilla trapped in a calcium sensitive luciferin binding protein. The released coelenterazine then reacts with molecular oxygen at the luciferase active site yielding blue light, CO₂, and coelenteramide. The Renilla luciferase is a 35-kDa protein, its cDNA has been cloned into *E. coli* which expresses a 314-amino acid sequence.

The luminescent shrimp *Oplophorus gracilirostris* secretes a cloud of luminescence from the base of its antennae and also has luminous organs on the legs. The mechanism underlying Oplophorus luminescence involves the oxidation of coelenterazine with molecular oxygen catalyzed by its multimeric luciferase yielding coelenteramide, CO₂ and the emission of 454 nm blue light (Shimomura et al. 1978). The Oplophorus luciferase is highly stable, with maximum luminescence occurring at 40°C. Later on, it was found that Oplophorus luciferase can catalyze light emission from a broad spectrum of coelenterazine analogues and its coelenterazine luminescence is considerably brighter than aequorin or the Renilla luciferase (Inouye and Shimomura 1997). The multimeric luciferase is composed of 19 kDa and 35 kDa proteins, expression of both cDNAs revealed that the luciferase activity is associated with the 19 kDa protein although stability and brightness are greatly improved when both proteins are coexpressed (Inouye et al 2000). Given that the Oplophorus luciferase is relatively small, heat stable, bright, able to use a broad spectrum of coelenterazine analogues as substrates, and is naturally secreted, it was an attractive luciferase for reporter genes. Hall et al. (2012) developed

an enhanced version of the 19 kDa protein through three phases, the first phase identified randomly generated mutants which enhanced the brightness of the reaction with coelenterazine, the second phase screened the pooled first phase mutant luciferase against 24 coelenterazine analogues to find substrates with increased luminescence and lower background noise. The third phase screened another set of random mutants of the already enhanced luciferase against coelenterazine and several analogues to find even brighter mutants. The result was the NanoLuc engineered luciferase, when used with the coelenterazine-analogue furimazine is 2.5 million fold brighter than the wild-type *Oplophorus* luciferase/coelenterazine (Hall et al. 2012).

1.3.6 Other terrestrial bioluminescence

Diptera from related families Mycetophilidae (*Orfelia fultoni*) and Keroplatidae (*Arachnocampa luminosa*) emit blue bioluminescence, but their mechanisms are poorly understood as luciferins and luciferases have yet to be discerned. Dipteran bioluminescence is likely independent of Coleoptera bioluminescence as the color of light is very different, known cofactors are different, and cross reactions among the systems does not result in light emission (Meyer-Rochow 2007). The diptera *A. luminosa* and *O. fultoni* appear to have different bioluminescent systems based on the specificity of different cofactors although there may be similarity at other levels as more information is obtained (Viviani 2002; Shimomura 2006). Recently, transcriptomes from luminous and nonluminous segments of luminescent *A. luminosa* larvae have been sequenced and compared, while the identity of the luciferase remains elusive, several candidate genes were reported (Sharpe et al. 2014, Silva et al. 2014).

In addition to beetles and flies, millipedes are another order of arthropods which are luminescent. Like the dipteran systems, millipede bioluminescence is far from understood. The blue-green light has a maximum wavelength of emission of 495 nm (Hastings and Davenport 1957). When dried and powderized millipedes are extracted with acetone, and the solvent

subsequently removed and replaced with water, cell free luminescence is observed for about 10 min. Addition of hot extract of millipedes to exhausted luminescing extracts does not result in additional light, thus the hot-cold extraction fails for millipede luminescence suggesting the possibility that the millipede system could be a photoprotein-like system. Additionally, it was found that ATP increases light emission and that FMN has an inhibitory effect. Shimomura, in two different works (1981, 1984) suggested possible luciferins for the millipede bioluminescent system, although both works are contradictory to each other and the results have yet to be verified.

1.4 The Current Biology of Fungal Bioluminescence

The majority of today's bioluminescent mushrooms are found throughout the tropical and temperate regions. The number of known bioluminescent mushroom species is approaching one hundred, with new species still being discovered (Dennis Desjardin, personal commun.). Fungal bioluminescence occurs only in the Phylum Basidiomycota in the Order Agaricales of gilled mushrooms, which is the largest clade of mushroom forming fungi and includes over half of the known mushroom species (Hibbett et al. 1997, Hibbett 2006). The over 9000 Agaricales species are distributed among six clades (Matheny et al. 2006). Less than 1% of the known Agaricales are described as bioluminescent. Bioluminescent fungi are not monophyletic and exist in four distinct lineages: Omphalotaceae, Marasmiaceae, Physalacriaceae, and Mycenaceae (Desjardin et al. 2008, Matheny et al. 2006). The first three lineages belong to the Marasmioid clade, while Mycenaceae belong to the Tricholomatoid clade.

The Omphalotaceae contain some of the largest and brightest bioluminescent mushrooms including the “Jack-O-Lantern” mushroom (*Omphalotus olearius*) (Hughes and Petersen 1998) and “Flor-de-Coco” (*Neonothopanus gardneri*) (Capelari et al. 2011). Extracts

of *N. gardneri* yield intense light when used in the in vitro light reaction and are the staple species of our lab.

Eleven *Armillaria* species are known to have luminescent mycelium (Dennis Desjardin, personal commun.). *Armillaria* species are commonly called Honey Mushrooms and are quite edible but not luminescent, but the ground mycelium is luminescent. *Armillaria* species are slow growing but known to cover great area with individuals covering hundreds of acres (Mihail and Bruhn 2007). Recently the genomes of three luminescent species have been sequenced, *A. gallica* (Grigoriev et al. 2012), *A. mellea* (Collins et al. 2013), and *A. ostoyae* (Grigoriev et al. 2012).

The marasmioid *Gerronema viduolum* found in the Brazilian Atlantic Rainforest (Mata Atlântica), is phylogenetically in its own clade of bioluminescence separate from the omphaloid clade and the mycenaceae clade. *Gerronema* was first described in 2005 (Desjardin et al. 2005), its culturing conditions determined for optimal mycelium light emission (Mendes et al. 2008) and was used by Oliveira and Stevani to confirm the enzymatic basis of fungal luminescence (2009).

The more distantly related lineage of bioluminescent fungi, Mycenaceae contains many smaller, yet still very bright mushrooms. *Mycena luxaeterna*, one species of many found in the Brazilian Atlantic Rainforest, produces intense light from its stipe whereas its cap and mycelium cultures are typically non-luminescent with naked eye (Desjardin et al. 2010). *M. luxaeterna* is the main mushroom of interest in the ecological studies and is discussed more throughout the thesis ecology sections. *Panellus stipticus*, described in more detail above, is more closely aligned with the Mycenaceae (Desjardin et al. 2005, Matheny et al. 2006).

Figure 2 The 3-hydroxyhispidin biosynthesis pathway: the 3-hydroxylation of the hispidin catechol group by a coumaroyl 3-hydroxylase (C3H) occurs prior to the addition of coenzyme A by 4-coumaroyl:CoA ligase (left side), or following bisnoryangonin formation (right side).

1.5 The metabolic pathway system of bioluminescence in fungi

As mentioned before the fungal luciferin has recently been identified as 3-hydroxyhispidin, which is biosynthesized from the stable luciferin-precursor hispidin. Hispidin is a polyketide styrylpyrone class compound that has a 13-carbon backbone composed of a catechol moiety and a 4-hydroxy-2-pyrone heterocyclic ring. Hispidin was first identified from extracts of the medicinal mushroom *Inonotus hispidus* which had been used traditionally to treat many conditions such as gastrointestinal cancer, cardiovascular disease, tuberculosis, liver or heart disease, bellyache, diabetes and other ailments (Lee and Yun 2011). Hispidin and its dimers have been shown to have potent antioxidant activity, scavenging free radicals in a concentration dependent manner (Jung et al. 2008). Hispidin is also known to be mildly cytotoxic, inhibiting isoform-Beta of human protein kinase C that is more cytotoxic in cancerous cells than normal cells (Gonindard et al. 1997). Hispidin has also demonstrated anti-viral activity against several types of influenza virus and as a potent inhibitor of HIV-1 integrase (Awadh et al. 2003, Singh et al. 2003). Hispidin also has been shown to be a potential tool against Alzheimer's disease as it is an inhibitor of BACE1 that cleaves β -amyloid products being a major component of amyloid plaques (Park et al. 2004).

Hispidin biosynthesis (Fig. 2) and the biosynthesis of other styrylpyrones have been extensively studied and is well characterized in polyporus mushrooms from the *Inonotus* genus and the ancient ferns of the genus *Equisetum* commonly called horsetail, which has an abundance of glycosylated hispidin (Fig. 1G) derivatives in their strobil (Veit et al. 1993). Styrylpyrone biosynthesis is part of the phenylpropanoid biosynthesis (KEGG:ko00940) a subsection of the larger phenylalanine metabolism (KEGG:ko00360). Phenylalanine secondary metabolism is highly conserved in plants and fungi, with homologous and analogous steps

common to both kingdoms and many metabolites and phenolic products including flavonoids, isoflavonoids, lignins, suberins and coumarins.

1.5.1. Phenylalanine ammonia lyase

The first committed step of phenylalanine secondary metabolism, is the deamination (NH_3 loss) of phenylalanine by phenylalanine ammonia lyase (PAL, E.C. 4.3.1.24), yielding trans-cinnamate (Nambudiri et al. 1973, Hanson and Havier 1981), whose process is spontaneous and only requires the phenylalanine substrate for the reaction (Koukol and Conn 1961). PAL is found in all green plants, most Basidiomycete and Streptomycete fungi (Camm and Towers 1973). PAL often occurs in multiple isoforms in plants and fungi (Lois et al. 1989, Kim et al. 2001, Zhao et al. 2015, Weign et al. 2013), both *I. hispidus* and *A. bisporus* have two isoforms of PAL. In both fungi and plants, PAL activity is stimulated by light (Nambudiri et al. 1973, Lois et al. 1989, Lodgeman et al. 1995).

1.5.2. Cinnamate 4-hydroxylase

Cinnamate Hydroxylase, also known as trans-cinnamate 4-monooxygenase (C4H, EC 1.14.13.11) converts cinnamate to *p*-coumarate. C4H is a NADPH-dependent cytochrome p450 gene and in plants belonging to its own family, CYP73 (Nelson et al. 1996, Teutsch et al. 1993). Plant C4H was first cloned from tuber tissues of Jerusalem artichoke (Teutsch et al. 1993), and many other plant species contain this enzyme. Studies directly focusing on C4H in fungi are limited, but many fungi contain genes with high sequence similarity to the plant genes, *A. bisporus* contains two hypothetical C4H genes (Weign et al. 2013). Early studies on C4H activity in fungi were in direct relation to hispidin, it was found that C4H activity in *I. hispidus* was dependent on NADPH and that maximum activity required FAD, FAD has not been shown to be required in higher plants for maximum activity (Vance et al. 1973). Vance et al. (1973) suggested that C4H activity in fungi is stimulated by light, later others verified this induction in

polyporus fungi (Nambudiri et al. 1973, Vance et al. 1974), numerous reports indicate that C4H is also stimulated in plants by light (Jin et al. 2000, Lodgeman et al. 1995).

1.5.3 Coumaroyl 3-hydroxylase

The most cryptic step of the biosynthesis of hispidin in both plants and fungi is the 3-hydroxylation of the hispidin catechol moiety. In fungi it was observed that the 3-hydroxylation of *p*-coumarate, like previous steps is stimulated by light (Nambudiri et al. 1973, Vance et al. 1974). Nambudiri et al. (1974) characterized two *I. hispidus* soluble proteins capable of performing 3-hydroxylation of phenylpropanoid rings, the first extracted protein with a molecular mass of 185 kDa was able convert *p*-coumarate to caffeate and bisnoryangonin to hispidin, similarly the second smaller 45 kDa protein was able to hydroxylate both substrates. In addition to the 3-hydroxylase activity the 185 kDa enzyme also had caffeate oxidase activity while the 45 kDa protein was only able to perform the forward reaction. Both proteins used NAD(P)H as electron donors and had almost identical 3-hydroxylase activities. Attempts in plants to identify the responsible enzyme, subsequent gene identification and cloning using free *p*-coumarate and cellular extracts yielded vague results and often low or completely absent quantities of caffeic acid. The enzyme responsible for 4-coumarate-3-hydroxylase (C3H) activity was identified in plants simultaneously by two different groups each employing two different identification strategies (Schoch et al. 2001, Frankie et al. 2002). Schoch et al. (2001) first identified orphan p450 genes with no known function in the Arabidopsis genome and through the analysis of p450 phylogeny identified the CYP98 family as being closely related to C4H (CYP73), the gene responsible for the first hydroxylation. Expression of CYP98 in yeast and screening with conjugates of *p*-coumarate found that CYP98 indeed catalyzed the 3-hydroxylation of the phenylpropanoid ring of 5-O-shikimate and 5-O-D-quinic acid both esters of *p*-coumarate into their caffeic acid conjugates, but in their study CYP98 was unable to form caffeic acid from

p-coumarate. Frankie et al. (2002) took a genetic approach to the identification of the 3-hydroxylase by screening leaves of arabidopsis mutants for a decrease in fluorescence by soluble phenylpropanoid secondary metabolites. The identified mutants were then fed with radiolabeled phenylalanine, the *ref8* mutant lacked labeled caffeic acid. Mapping of the *ref8* mutant identified a p450 gene in the CYP98 family, the gene was expressed in yeast yielding a 58 kDa protein which was screened against *p*-coumarate and related analogues. Caffeic acid was detected when *p*-coumarate was incubated with CYP98, but caffeic acid yield was below the required level for detailed kinetic analysis. CYP98 had greater activity with the methyl ester of *p*-coumarate. Both studies identified CYP98 as the plant C3H and that *p*-coumarate is not the preferred substrate, suggesting that *p*-coumarate is not directly converted to caffeic acid but instead *p*-coumarate is shuttled through other levels of phenylpropanoid metabolism where it is 3-hydroxylated by CYP98, and then free caffeic acid is subsequently released, such as the shikimate pathway.

1.5.4 4-coumaryl:CoA-ligase

The final step in the phenylpropanoid biosynthesis pathway is catalyzed by a hydroxycinnamyl:CoA-ligase, more commonly referred to as 4-coumaryl:CoA-ligase (4CL, EC 6.2.1.12) acting as a gateway for phenylpropanoids to flavonoid, isoflavonoid, lignin, suberin, coumarin and phenolic metabolic pathways. Substrates of 4CL are CoA, ATP, and hydroxycinnamate the reaction yields AMP, pyrophosphate and hydroxycinnamoyl-CoA products. In general 4CL is a promiscuous protein, accepting various hydroxycinnamates as substrates including 4-coumarate, caffeate, and cinnamate, although substrate specific cinnamate:CoA-ligases also exist in plants. 4CL is often encoded by several different genes in plants, in some species the resulting proteins are nearly identical with the same substrate preference, while in others the different isoforms show substrate preference and spatial

expression patterns associated with different downstream pathways (Ehlting et al. 1999). The hispidin synthesizing fungus *I. hispidus* contains active 4CL, the partially purified enzyme fraction is capable of catalyzing the formation of CoA thiol esters of *p*-coumarate, caffeate, and ferulate, consistent with 4CL substrate promiscuity observed in plants (Vance et al. 1975). 4CL in *I. hispidus* is light activated as extracts from dark grown mycelial cultures are unable to synthesize caffeoyl-CoA and feruloyl-CoA, and has a 80% reduction of *p*-coumaryl-CoA synthesis. Two isoforms of 4CL were identified through bioinformatic means in the hispidin producing *I. obliquus* polyporous fungus, both 4CLa and 4CLb were cloned, expressed and expression levels monitored in response to coculture of *I. obliquus* with a competing fungus *P. morii*. In response to this stress, 4CL and other enzymes related to phenylpropanoid metabolism were upregulated through likely a nitrous oxide dependent signaling pathway (Zhao et al. 2015).

1.5.5 Polyketide synthase

Perrin and Towers (1973) demonstrated that the pyrone moiety of hispidin and bisnoryangonin are formed from the addition of radiolabeled acetate units from labeled malonic acid, although in their study the enzyme catalyzing the reaction was not well characterized. Similar results were found for hispidin biosynthesis in *Equisetum arvense*, where a 56-77k Da protein was purified that produced bisnoryangonin when *p*-coumaric acid and malonyl-CoA were supplied and termed styrylpyrone biosynthesis (Beckert et al. 1997). The activity of styrylpyrone synthase is similar to that of chalcone synthase, a type III polyketide synthase, which is the initial step of flavonoid biosynthesis. Interestingly, in *E. arvense*, styrylpyrone-glycosides are the only polyketide compounds found in the gametophytes and rhizomes with no flavonoids detected while in the green sprouts flavonoids are found but no styrylpyrones (Veit et al. 1995). Chalcone synthase generally catalyzes three condensations reactions each incorporating an acetyl group derived from the decarboxylation of malonyl-CoA, while styrylpyrone synthase

performs only two condensation reactions. Polyketide synthases are relatively well characterized (Hertweck 2009, Abe and Morita 2010, Weng and Noel 2012, Rawlings 1999), there are three main families, type I, II, and III, type III is the main type in plants with chalcone synthase the most characterized. All three polyketide synthase families likely evolved from enzymes in fatty acid primary metabolism, with which they share overall structure and mechanism. Fatty acid chain elongation in *E. coli* is achieved by the activities of three different ketoacyl synthase enzymes, type I, II, and III. Type I ketoacyl synthase elongates C4 fatty acids to C16 fatty acids, Type II elongate C16 to C18, and type III initiates this process elongating C2 acetyl-CoA to a C4 intermediate. All three ketoacyl synthases elongate fatty acids from C2 units by repeated head-to-tail linkage, the starter acyl unit is condensed with a malonyl unit that undergoes decarboxylation to provide electrons for the new carbon-carbon bond resulting in a β -keto ester which is then reduced to a hydroxyl, dehydrated forming an enoyl, and then reduced again to form a saturated chain two carbons longer than the starting acyl. All three ketoacyl synthases at minimum contain a ketosynthase domain. Type I ketoacyl synthase is a modular protein with the ketosynthase, acyl carrier protein, ketoreductase, dehydrogenase, and enoyl reductase in separate domains each with their own active site. Type I ketoacyl synthase forms a polyprotein complex with similar type I ketoacyl synthase which act successively to elongate the growing fatty acid, but each sub-protein contains all the functional domains for the cycles of fatty acid elongation it catalyzes. On the other hand Type II ketoacyl synthase is similar to Type I but is instead a multi-protein complex with active domains distributed among the different proteins, Type II ketoacyl synthase complexes often dimerize to form even larger complexes as Type I ketoacyl synthases. Type III ketoacyl synthase composed of a single protein like type I but instead of having separate sites for the ketosynthase, ketoreductase, dehydratase, and enoyl reductase steps, these activities occur in a single active site. Type I

polyketide synthase resembles Type I ketoacyl synthase, both are multi-domain proteins that form polyprotein complexes, similarly Type II polyketide synthase resembles type II ketoacyl synthase and type III polyketide synthase resembles type III ketoacyl synthase. The greatest mechanistic difference between polyketide synthases and their ketoacyl synthase ancestors is that ketoacyl synthase produces completely reduced (C-H, C-C bonding) products, whereas polyketide synthase yields unsaturated and hydroxylated bonds, the degree of unsaturation dependent on the activity of ketoreductase, dehydratase and enoyl reductase domains. Polyketide synthases are much more variable than their ancestor and can act on varying carbon units of the growing molecules allowing for ring formation and branching to occur. Polyketide synthases can catalyze three types of ring formation, claisen, aldol, and lactone. Polyketide biosynthesis is diverse depending on the given polyketide synthases specificity of starting compound, degree of elongation steps and type of ring formation. All three polyketide synthase families can be found in bacteria, but in higher organisms the occurrence is not uniform. Plants typically have type III polyketide synthases. The majority of type I polyketide synthases have been found in fungi and animals although more recently type III polyketide synthases have been found in some ascomycete fungi at a much a much lower prevalence (Hashimoto 2014).

1.6. Related chemistries

Hispidin biosynthesis in *I. obliquus* is stimulated by light through an increase in activity of phenylalanine ammonia lyase, cinnamate hydroxylase, and *p*-coumarate hydroxylase (Nambudiri et al. 1973, Vance et al. 1974). In ethanolic extracts of *Inonotus* that contain hispidin another secondary metabolite, hispolon (Fig. 1H), is often found (Ali et al. 1996). Hispolon is a twelve carbon phenylpropanoid that has many structural similarities to hispidin (Balaji et al. 2015). It has not been established if hispolon is a direct byproduct of hispidin metabolism or from the alternative activities of hispidin precursors. In addition to the smaller hispidin and

hispolon products, larger polyketide products with similar catechol and pyrone moieties are also found in these polyporus mushrooms such as isoscavin A (Fig. 1I).

The major styrylpyrone in horsetail (*Equisetum* genus) is 3-glycosyl-hispidin termed equisetumpyrone, which can be extracted in substantial quantities from the gametophyte and roots (Veit et al. 1993). Horsetail has two forms of type III polyketide synthases, styrylpyrone synthase (SPS) which forms hispidin with two malonyl-CoA subunits and expressed in the gametophyte, the other a chalcone synthase (CHS) found in the stems which performs three malonyl-CoA additions. The horsetail CHS likely evolved from a duplication of its own two-step SPS gene as (Veit et al. 1993, Beckert et al. 1997). Chalcone synthases are common in plants. Both the *Inonotus* and the *Equisetum* styrylpyrone synthases are general hydroxycinnamic acid malonyl-CoA transferases.

1.7 Function and ecology of bioluminescence

It remains unknown what additional function, if any, bioluminescence serves to fungi. Most other bioluminescent organisms use the light by-product for some additional function that involves specialized biochemistry, morphology, physiology, and behaviors. Generally bioluminescence is thought to function as an attractant or repellent: long steady bioluminescence can be attracting to curious critters but can also be used as aposematism to repel, while short flashes are thought to attract over longer distances and repel at close range (Haddock 2010). It is likely that bioluminescence evolved under selective pressure of the visual interactions between predator and prey in most cases (De Cock and Matthysen 1999).

The bright and rapid flash of disturbed dinoflagellates is one of the best studied cases at the ecological level. When water, rich in dinoflagellates, is disturbed, secondary predators are attracted to the area and can eat the primary predators of dinoflagellates resulting in lower rates of dinoflagellate predation and higher dinoflagellate density (Abrahams and Townsend 1993,

Mensingher and Case 1992, Fleisher and Case 1995). Elephant seals and sperm whales have been observed disturbing dinoflagellate blooms in order to lure prey to the area (Campagna et al. 2001, Frstrup and Harbison 2002), whether or not this has any effect on dinoflagellate fitness is unknown but it does affect the ecology. Edith Widder described blooms of bioluminescent biota as “bioluminescent minefields” sensitive to animal movement (Widder 2001). The other functions of bioluminescence in the marine environment are wide, diverse and far from being fully studied. These include sacrificial luminescent limbs, tagging translucent predators, distractive secretions, camouflage, lures, illumination/motion detection, communication, and surely other unknown functions.

Terrestrial bioluminescent arthropods have gained numerous additional functions of bioluminescence for both attraction and repulsion. Fireflies, and other bioluminescent beetles, display complex light patterns for species specific communication. Different species of fireflies have different temporal patterns and wavelengths of light emission for the attraction of mates (Buck and Case 2002, Carlson and Copeland 1985). Females of the firefly genus *Photuris*, known to be carnivorous, mimic the flashing code of the females of the genus *Photinus* in order to attract *Photinus* males and then devour them (Lloyd 1984, Eisner et al. 1997, Gonzalez et al. 1999). It has been speculated that the slow steady light of the luminescent larvae of the diptera *Mycetophilidae*, are used to lure prey into their sticky webs and repels negatively phototactic predators (Sivinski 1998, Gatenby 1959). On the other hand, the luminescent larvae of fireflies are mobile and actively hunt for their prey, instead of using luminescence as a lure it is likely that luminescence serves a defensive aposematic role as *lampyridae* are often unpalatable to many insectivores and some animals actively avoid luminescent insect prey (De Cock and Matthysen 1999). Aposematism has also been observed as a likely function of millipede bioluminescence, rodents attacked non-luminescent millipedes and non-luminescent millipede clay models

significantly more than luminescent samples (Marek et al. 2011).

Similar ecological patterns may be found from the study of fungal luminescence ecology. Luminescent fungi being immobile, mostly saprotrophic white-rot decomposers of plant material, and emitting a constant glow, likely excludes the predatory attraction of prey and the attraction of mates as likely functions, but attraction could still be an important function to Fungi. Sivinski (1981) performed the last known ecological study of fungal luminescence. One observation was that arthropods known to prey on other arthropods were found associated with luminescent fungi in numbers bordering on significance. Sivinski proposed several hypotheses as to the ecological function and evolutionary significance of fungal bioluminescence.

Attraction of insectivores to fungi could be acting similar to the burglar alarm effect, where spiders and other predators prey upon fungivores and other arthropods attracted to the mushrooms. It is very unlikely that luminescence serves some intra-species communication function during mating, although it could be an efficient means to attract spore dispersers. Insects have been reported as vectors of fungal propagules, and some fungi have specialized visual cues and chemical odors to attract insects. This relationship has been observed with flies and stingless bees that defecate viable spores after ingesting the gleba of stinkhorn mushrooms (Ramsbottom 1953, Tuno 1998, Oliveira and Morato 2000); the closely linked life cycles of the woodwasp *Sirex noctilio* and its fungal symbiont *Amylostereum areolatum* (Slippers et al. 2012); and, bark beetles that transmit the fungus responsible for the Dutch Elm disease (Webber 2000). Aposematism is a very likely function of luminescence, repulsion of fungivores by the luminescent light would likely have the strongest impact on the fitness of luminescent fungi. Insects could be repelled by green light because of neophobia, innate bias, or learned avoidance. The pressure on these insects to avoid this color light could be due to the association between the light and the unpalatability; toxicity of some luminescent mushrooms

such as *Omphalotus olearius* (Vanden Hoek et al. 1991); because of the association of green light with luminescent insects that are either predators or are unpalatable.

Fungal propagules could be spread by passing through the gut of the insect or adhered to the exoskeleton. Basidiomycete fungi require help in getting from place to place to colonize new substrates; most achieve this through the use of winds that can carry light weight spores and others are aided by animals for diaspore dispersal. In the case of spore dispersal by arthropods, it can occur by the transportation of spores adhered to the body (ectozoochory) or inside the gut of the arthropod (endozoochory) (Lilleskov and Bruns 2005). Moreover, Basidiomycetes spread their spores during the day and night, but at night hyphal germination is higher as both substrate and spores are more hydrated. Hence, it is reasonable to assume that nocturnal dispersion of spores by insects might be more effective and advantageous to fungi especially in dense forests.

Many arthropods are known to be phototropic: bug zappers and street-lights serve as common examples. The basic trichromatic vision system in insects dates back to the Devonian era ancestor of all winged insects (Briscoe and Chittka 2001). The common vision system is UV-blue-green sensitive although there is great variation of this pattern that allows for different insects to sense light of different intensities and of different wavelengths. Most insects studied have green receptors maximally sensitive at ca. 530 nm, UV receptors with maximum sensitivity at ca. 350 nm, and most species have blue receptors with maximum sensitivity at ca. 440 nm (Briscoe and Chittka 2001). Although more rare, some insects have red receptors (ca. 565 nm), the longest maximum sensitive wavelength recorded is from the Glaphyrid beetle with a maximum wavelength of 630 nm (Briscoe and Chittka 2001, Martinez-harms et al. 2012). The study presented in thesis is chiefly concerned with two wavelength regions: green and infrared. Bioluminescent fungi emit green light with a maximum wavelength of emission of ca. 535 nm,

therefore if there is a relationship between arthropods and fungal bioluminescence it would be expected that the arthropods are sensitive to this wavelength, which many are. Secondly, the infrared, we employed the use of infrared video for monitoring the interaction of arthropods and the bioluminescent fungi, it is important that insects are not sensitive to the infrared light (700 - 1000 nm), luckily the highest observed red light sensitivity of any insect is 630 nm, well below the infrared level.

2. Objectives:

The main objectives of this project was to a) identify and clone the fungal luciferase and further progress the molecular understanding of fungal bioluminescence, and b) additionally to better understand the evolutionary significance of this trait in fungi through field studies.

More specifically the objectives of this project were:

- Further purify the fungal luciferase protein
- Explore fungal luciferin biochemistry
- Investigate the genomics of fungal luminescence and luciferin biosynthesis
- Glue trap and infrared monitoring of *Mycena luxaeterna* and *Neonothopanus gardneri*

3. Methods:

3.1. Field studies:

3.1.1 Locations and fungi

Ecology experiments were conducted in two different ecoregions of Brazil, the Atlantic Rainforest in São Paulo State and in the transitional Coconut Forest of Piauí (Mata dos Cocais). The field sites in São Paulo State are located inside the Parque Estadual Turístico do Alto Ribeira (PETAR) and at a private nature center Instituto de Pesquisa da Biodiversidade (IPBio), a NGO whose area is located along the Betari river. This area is characterized by having tropical moist forests with low altitude but steep mountainous terrain. The Atlantic Rainforest is

one of the most biodiverse regions of the world, with over thirteen different tree families (Saia et al. 2008, Ivanauskas et al. 2012). Bioluminescent mushrooms can be found throughout the forest in this area. The field site at IPBio was selected due to the reliable and abundant occurrence of the *Mycena luxaeterna* mushroom as well as several other less common bioluminescent mushrooms species. *Mycena luxaeterna* (Fig. 3) is a ca. 5 cm tall mushroom with luminescent stipe and typically non-luminescent cap. *M. luxaeterna* can be found on several plant substrates including *Euterpe edulis*, *Miconia* sp. *Jacaranda micrantha*, *Schizolobium parahyba*, and *Inga* sp. In this specific location it is not uncommon to find over 75 luminescent mushrooms in a single night when conditions are conducive, which is significantly greater than any other area where we have observed this species. This specific location is a protrusion of riverside that is about 5 m above the river and is surrounded on three sides by the river, steep mountainsides surround the general area.

Experiments were also performed in the Northern state of Piauí in the Mata dos Cocais (Coconut or Babaçu Forest). This ecoregion is a direct northern extension of the savannah-like Brazilian vegetation called Cerrado that characterizes the interior of Central Brazil. Piauí's Babaçu Forest straddles the very wet Amazon Rainforest to the west and the arid Caatinga to the east (Castro et al. 1998). *Neonothopanus gardneri* (Fig. 3) is the only bioluminescent mushroom observed in this region, it is one of the largest and brightest bioluminescent mushrooms and can be found easily due to its brightness and abundance during the wet season (Capelari et al. 2011). The *N. gardneri* mushroom is only found on young Babaçu palms in the test area known as "pindoba". Mushrooms are typically found growing on the base of the midrib of senescing palm fronds that are still attached to the plant and that are in contact with the soil surface, it is not clear if the mushroom induces senescence or depends on the senescent frond. Mushrooms cannot be found on older palms where the trunk separates live fronds from the soil

nor are they found on fallen fronds that cover the forest floor. Palms with mushrooms are typically found in close proximity (< 100 m) to either a creek (> 5 m) or a vernal pond, palms further away from water in noticeably drier areas lack mushrooms. The ground mycelium of *N. gardneri* is robust in the area, on dry nights it cannot be easily seen, but following a heavy rain and then disruption of the soil surface, by foot traffic, will result in a more than detectable glow.

Figure 3: Glue trap apparatus. Top, glue trap style used in the transitional palm forest next to *N. gardneri* mushrooms. Middle, *M. luxaeterna* mushrooms on fallen *Inga* sp. branch. Bottom, glue trap style used in the Atlantic Rainforest.

3.1.2. Encountering Bioluminescent Mushrooms

Bioluminescent mushrooms can be easily found in the forests if certain conditions are met. Firstly, eyes must be dark adapted, this can be achieved by turning off all lights for at least 1 min, preferably 5 min of dark adaptation. The phase of the moon must also be taken into account, while the bioluminescent mushrooms can be seen in full moonlight the task is complicated as bleached decaying leaves shine quite bright on the forest floor which can obfuscate the detection of mushroom light. The darkest conditions are found on new moon nights, up to about seven days prior to the new moon are also conducive as the crescent moon rises in the early morning often after nightly excursions are completed, also several days following the new moon moonless conditions can be achieved following the moon set which occurs about 70 min later each successive night. When these dark conditions are met the only light seen under a forest canopy is bioluminescent light, either insect bioluminescent light or fungal light. In some locations where bioluminescent mushrooms are found the forest floor can also be illuminated by ground mycelium, which is brightest and most easily seen following or during a rainstorm and can be made brighter by scraping the soil by foot.

Bioluminescent mushrooms are not uniformly found in all forests or within a forest known to have bioluminescent mushrooms. To find new locations one must navigate the forest under darkness, *night-hiking*, keeping lanterns defaulted to the off setting. Nimble navigation can be sped up, and thus more area explored, by the adaptation of several techniques. At the heart is navigating existing and preferably well maintained forest trails, instead of visually following the trails the trails can be sensed by foot. Dragging one's feet along the trail can minimize tripping. Further, hiking trails are often mildly convex in shape, the trail edges can be sensed by increased roll angle under foot as movement direction deviates from trail direction. Wandering off the trail can also be minimized by sound, well used trails are often more free of debris than

off-trail, the crunch of footsteps on and off trail also sounds different. The additional use of a hiking stick (a fallen tree branch works fine) about 125% the length of the user's height also greatly facilitates dark-hiking, bouncing the the ground end of the hiking stick about 1m in front of the nimble navigator can help detect trail direction by feel and sound. The height of the hiking stick kept vertical and at arm's length in front of the navigator also clears spiderwebs and detects other objects blocking the trail. Walking in the forest at night in almost total darkness is far from impossible, with these techniques mastered one can walk and thus cover new locations at an almost normal pace while maintaining total eye dark adaptation, further, navigating by feel and sound frees the visual sense to focus on detecting light.

When a bioluminescent mushroom is encountered while dark-hiking on-trail the surrounding area off-trail should be explored as often mushrooms are found in patches. Walking off a hiking trail is often the first step to getting lost in the woods, disorientation is exacerbated by the darkness. Sound, again, can help minimize disorientation. When exploring in a group, maintaining vocal-communication can provide a general sense of position between group members, this can be achieved by talking at short range or by making a loud “WOOOP” noise and reciprocating the “WOOOP” when heard. When exploring off-trail alone, losing the trail is very easy, the simplest solution to this is playing music on a portable music device with the volume setting set to eleven. Place the music device on the trail, one can freely explore the surrounding area as long as the music can be heard without getting disorientated.

3.1.3 Glue trap arthropod collection and analysis:

The glue trap experiment (Fig. 3) to study animal attraction to fungal luminescence is similar to the one performed by Sivinski (1981), which involved a similar glue on glass vials containing luminescent mushrooms but differs in one noteworthy manner: we used artificial LED light instead of natural light. A petroleum based insect glue (TangleFoot, Tangle-Trap®) was

used to capture animals that landed on the faux mushrooms. The light source was a green LED (~ 530 nm) driven by a battery bank (3.7V, 1000mAh) and resistors (0.5 MΩ) to dim the light. In the *M. luxaeterna* experiment the acrylic mushroom facsimile had a thin stipe and small cap 1 cm diameter, standing, 4-5 cm tall. Three facsimiles were attached to LED lights protruding from a small battery holder. In the *N. gardneri* experiments the acrylic mushroom facsimile was attached directly to the base of young Babaçu palms about 10 cm off the ground, a LED was placed directly in the base of the acrylic mushroom and connected by wire to the battery pack placed on the ground about a half meter away. Control traps were simply the same apparatus as above but without the LED light turned on. At sundown LED traps and control traps were randomly placed in the field sites on a macro scale, but positioned in a manner similar to how the mushrooms are naturally positioned. Insects were also directly collected from wild mushrooms when possible. The following sunrise glue traps were collected, all pieces of matter were removed from the glue with fine tipped forceps, debris discarded, and arthropods were preserved in 75% ethanol for later identification. Fresh glue was applied on top of trap surfaces following debris and insect removal. Arthropods stored in ethanol were first cleaned in several washes of hexane to remove excessive glue carried over into storage. Cleaned arthropods were then separated based on arthropod order and counted. Orders with abundant representatives were then further identified to the family level by Entomologist Prof. Silvio Nihei (IB-USP). Arthropod counts from control traps were compared to LED traps by means of a t-test. Glue trap experiments were performed in 2012 and 2013 in both the Atlantic Rainforest and Piauí.

3.1.4. Infrared monitoring and analysis:

In addition to the glue LED traps, direct infrared video observations of animals with the luminescent mushrooms were also performed. Infrared footage of mushrooms were acquired with four modified GoPro Hero3® white edition cameras. Initial modifications included

replacement of the stock lens with an aftermarket lens without the IR filter, a new 3D printed housing with an infrared LED light bank for illumination and a large capacity external battery (Anker, 5V, 10Ah). In the second year of observations the original camera housing was used, which allowed for easier manual focus and the LED light array was redesigned for improved and more disperse illumination of the visualized area. Mushrooms were documented by 5MP image timelapse with 0.5s intervals. Image acquisition continued until the cameras were collected the following morning or the batteries died, typically 8-10 h of footage was captured per camera per night. Images were viewed and annotated using custom built software using the openCV python interface (Bradski 2000). This annotator synced the current viewing frame of time lapse video with automatic annotation log file generation, while video still has to be manually observed, the generation of the log file was automated based on identified arthropods and the frame count. The resulting log file can be easily examined or parsed to extract temporal activities of arthropods around the bioluminescent mushrooms. Infrared experiments were performed in 2014 and 2015 in both the Atlantic Rainforest and Piauí. Control Infrared timelapse sets selection is discussed in more detail in the results and discussion sections.

3.2 Laboratory studies:

3.2.1 Mycelium Cultures:

Mycelium cultures are essential for a ready supply of fresh fungal biomass. Mycelium cultures can be initiated from mushroom basidia on agar media (BD Difco, potato dextrose agar: 4 g/L potato starch, dextrose 20 g/L, agar 15 g/L, autoclaved at 121°C for 15 min) cooled to about 60°C then antibiotics added, chloramphenicol (25 µg/mL) and phenylphenol (5 µg/mL), media is then poured into 60mm plastic petri dishes (Stamets 1993). For smaller mushrooms, the basidia is sliced into small 2 mm² segments, each segment is picked up with forceps, dipped into a mild bleach solution (5%) and rinsed in distilled water, this is repeated two additional times

before placing the segment in the center of the PDA petri dish. For larger mushrooms, mycelium is collected from the center of the mushroom by slicing the stipe in half, and cutting a small freshly exposed mass and placing in the center of the PDA petri dish. Agar petri dishes are then sealed with PVC film and incubated at 27°C. Cultures are monitored daily for observance of mycelium growth and removal of plates contaminated with stray bacteria or ascomycetes contaminants. Healthy mycelium is then used to inoculate fresh media after about 10 to 14 days.

Seven agaricales strains were used in this study *Neonothopanus gardneri*, *Lentinula edodes*, *Omphalotus olearius*, and four geographic isolates of *Panellus stipticus* (BL - Blount county, Tenn.,USA TU- Turkey, PK - Polk county, Tenn, and SW - Switzerland). Cultures were initiated in the field or provided by Dr. Dennis Desjardin (San Francisco State University), the *P. stipticus* cultures were provided by Dr. Ron Petersen (University of Tennessee). All fungal mycelium cultures were maintained on MYA media (5% black strap sugarcane molasses (82.2°Bx, Pol 56%. Usina São José da Estiva, Novo Horizonte, SP) or malt extract (80% malt solids, 8°Plato, 30°Lovibond. Briess CBW traditional Dark), 1% yeast extract, 2% agar) in 100mm petri dishes in the dark at 25°C. Stock cultures were subcultured once a month. Mycelium biomass for DNA, RNA, and protein extraction was prepared as above with the addition of a hydrated and autoclaved dialysis membrane placed on top of the solidified agar, which are inoculated with 7 evenly spaced 2 mm³ blocks of mycelium agar from stocks. Cultures are grown for 10-14 days before sheets of mycelium covered dialysis membrane are peeled from the agar media and used immediately for protein preparations or stored at -80°C following liquid nitrogen flash freezing until nucleotide extraction.

Backup stocks of mycelium were prepared from fully colonized 100 mm petri dishes by cutting the agar/mycelium into many 5 mm³ blocks and storing in 15 mL Falcon tubes filled with distilled and autoclaved water. Backup stocks were then stored at 4°C and were refreshed every

6 months, although they could be used to initiate new cultures for upto a year after preparation.

Light emission from mycelium cultures on agar petri dishes was measured by two main methods. The first being the Tecan Infinite M200 multi-well plate luminometer, with a custom built mount for four 5 cm petri dishes. Petri dishes were scanned with the 96 well plate setting with 1 s integration time, the resulting spreadsheet values were grouped for each petri dish and averaged. The second method for measuring light emission of mycelium cultures was with a GE ImageQuant LAS 4000 mini gel imager, images were generally captured with standard chemiluminescent imaging settings with 1 min integration time.

3.2.2. Luciferase enzyme extraction

Obtain around 10 *Neonothopanus* mycelium agar plates and remove the mycelium with the dialysis membrane. Then homogenize the mycelium with a motorized potter homogenizer in a tris/glycine buffer, typically about 1-2 g mycelium in 10 mL of tris-glycine buffer (100 mM tris base, pH 7.4, glycine 20 g/L). With the addition of glycine to the buffer the luciferase is quite stable and will stay active for a few months if extract is frozen. We normally homogenized with a potter homogenizer. Centrifuge 10 min at 10,000 x g to remove cellular debris. At this point if horsetail extract containing equisetum pyrone is added, abundant light should be observed. The cold extract can be further separated into three fractions by ultracentrifugation: 1) luciferase, 2) glucosidase and 3) hydroxylase. To separate the luciferase add to the crude extract CaCl_2 to 10 mM and incubate on ice or 4°C for 30 min, then centrifuge at 30,000 x g for 20 min - the luciferase should be pelleted, remove supernatant and solubilize the pellet in 1-2 mL of extraction buffer (tris/glycine). The glucosidase is in the supernatant, it can be further separated by ultracentrifugation for 1 h at 200,000 x g. It should be pelleted, the supernatant removed and the pellet solubilized with 1-2 mL of extraction buffer.

Luciferase can also be separated from the hispidin 3-hydroxylase by ultracentrifugation (1 h, 200,000 x g) of the cleared crude extract without the addition of CaCl₂. In this case the glucosidase is precipitated with the luciferase. Ultracentrifuged prepared luciferase was used in most luciferase enzymatic studies.

3.2.3. Luciferin extraction methods

Initially luciferin used in this project was obtained from hot aqueous extraction of dried and pulverized *N. gardneri* mushrooms, this was performed with a nitrogen purged Dionex® ASE 300 solvent extractor with aqueous solvent (20 mM 1,2-mercaptoethanol, 0.025% formic acid, in water). After it was known that hispidin is the hydroxylase substrate and 3-hydroxyhispidin is the luciferin, “hot-extract” solutions were made from pure samples (Sigma-Aldrich, and from Ilia Yampolsky) by dissolving and diluting in organic solvent (acetone, ethanol or DMSO) to prepare working solutions and storing by freezing or rotoevaporation with subsequent freezing. Working dilution solutions were typically prepared in water.

Bioluminescent active compounds can be extracted from some plants. Several Equisetum stands were found on the campus of USP and ornamentally in neighboring regions, and served as a source of horsetail strobili. Other plants were tested as well, such as guacatonga (*Casearia sylvestris*), water lettuce (*Pistia stratiotes*), *Arabidopsis thaliana*, several tissues of lime, and several species from the genus *Piper*, full list of tested plants in table 9. Plant tissue was flash frozen in liquid nitrogen and ground into a fine powder with mortar and pestle. The powderized material was then dissolved in either methanol, ethanol, or acetone, typically 1 g of material in 10 mL of liquid was incubated at on ice for 15-30 min. Cellular debris was cleared by centrifugation. The supernatant can be used directly in luciferase assays or can be further processed. Additional processing includes roto-evaporation to remove the solvent

and subsequent solubilization in organic solvents in the case of concentrating, or solubilization in water for some tests.

3.2.4. *In vitro* bioluminescence assay

The *in vitro* light bioluminescence assay is the predominant method for monitoring and quantifying components of the bioluminescent system. It involves combining the various protein cold extracts with hispidin and NADPH and observing light. The procedure involves the use of 50 μ L of cold protein extract, 50 μ L of hispidin, 50 μ L BSA (1 mg/mL), 50 μ L of NADPH (1 mg/mL). For assays in which the reductase and the luciferase are separated, 50 μ L of supernatant of the 100,000 x g ultracentrifugation, 50 μ L of the resuspended pellet, 50 μ L of the hot extract, 50 μ L BSA (1 mg/mL), and 50 μ L of NADPH (1 mg/mL) are mixed and immediately observed for light emission. Additionally, the actual luciferin, 3-hydroxyhispidin can react directly with the luciferase and does not require NADPH for light emission, 3-hydroxyhispidin can be used with either the crude protein extract or hydroxylase free ultracentrifuged pelleted luciferase.

In vitro light intensities were measured using either a Berthold DS Sirius tube luminometer, a Tecan Infinite M200 multi-well plate luminometer, or Berthold Sirius L tube luminometer. All three luminometers are more than capable of detecting fungal luminescence with standard extracts although the sensitivity of the Berthold Sirius L tube luminometer is superior. Light emission data was collected by two means, simple binary light or dark was monitored for up to 5 min with the digital readout, or 1 s intervals total light intensity measurements were recorded with timestamps and plotted for up to several hours but normally 15 min.

3.2.5. DNA extraction from mycelium

Several DNA extraction methods were tested but yielded low quantity and/or low quality highly pigmented DNA: Qiagen DNeasy, Promega genomic DNA kits, Invitrogen genomic DNA kits, SDS based methods, CTAB extraction, Sarkosyl based extractions. Ultimately, a new, novel and effective method of DNA extraction was developed. Prewarm a 10% CTAB (cetyltrimethylammonium bromide, Sigma-Aldrich) solution to 65°C. Grind 1 g of mycelium covered dialysis membrane into a fine powder with liquid nitrogen and a mortar and pestle, transfer powder to a 50 mL Falcon tube and add 10 mL of extraction buffer (BSA 1 g/L, 0.05 M Tris-HCl pH 8.0, 0.5 M EDTA), 100 μ L RNase (10mg/mL, Sigma-Aldrich), and 3.33 mL of 10% Sarkosyl (N-Lauroylsarcosine sodium salt, Sigma-Aldrich). Vortex briefly and Incubate for 1 h at 37°C to digest RNA, mix sample several times during incubation by hand. Centrifuge 5000 x g for 10 min to pellet cellular debris, transfer supernatant to new tube. Add 70 μ L of proteinase K (20 mg/mL) and 3.33 mL 5M NaCl, briefly mix and place in a 65°C incubator to warm. Add 10% CTAB solution in 0.5 mL increments until solution slightly thickens and phases begin separating, shake one more then keep vertical, incubate at 65°C. Usually 2.5 mL of 10% CTAB is sufficient for phase separation. If it is thought too much CTAB was added then add small volumes, less than 0.1 mL of 10% Sarkosyl until phases separate. Once phases separate, the lighter colored bottom phase should be about the same volume as the initial supernatant with a sharp interface between phases, if not adjust with CTAB and Sarkosyl, place biphasic solution into 4°C for 30 min without disturbing phases. Equalize the pressure of the tube by opening the cap, and prepare a new collection tube. Pierce the bottom of the biphasic tube and collect the lighter colored lower phase by gravity dripping into the collection tube, do not transfer any of the upper phase, discard the thick upper phase. Add 1/10th volume 3M sodium acetate mix and incubate at -20°C for 30min, centrifuge at 5,000 x g for 60 min, transfer supernatant to a new tube and avoid any precipitation or surface film. Add two volumes of absolute ethanol and incubate at

-20°C for 20 min to overnight. Centrifuge at 5,000 x g for 60 min and discard supernatant. Completely resuspend pellet in 5 mL water, this solution should be a cloudy white solution. Centrifuge at 5000 x g for 60 min, the supernatant should be transparent and a small pellet should have formed, avoid and discard this pellet. Add an additional 5 mL of 5M NaCl, and 20 mL 95% ethanol to the supernatant, incubate at -20°C for 1 h. Centrifuge at 5000 x g for 60 min at 4°C. Discard supernatant and completely resuspend pellet in 500 µL of water, then add 1 mL 95% ethanol. Centrifuge at 10,000 x g for 30 min. Continue to resuspend pellet in 500 µL water and then adding 1 ml 95% ethanol and centrifuging until the size of the pellet stops reducing in size, typically 3 washes total. The pellet is largely DNA at this point but it was found essential to first solubilize the pellet in 500 µL H₂O and centrifuge at 10,000 x g for 30 min, a small soft but firm glassy pellet should form at the bottom of the tube, which should be discarded, transfer supernatant to a fresh tube, precipitate DNA with two volumes of absolute ethanol and centrifuge for 30 min at 10,000 x g. Concentrated genomic DNA is then solubilized in a final volume of 50 µL in DNase free water or standard TE buffer. DNA for genome sequencing was quantified by spectrophotometry and Qubit, DNA integrity and ability to undergo enzymatic reactions were determined by running a digested and undigested 1 µg DNA sample on a 1% Agarose gel (TAE).

During the process of developing the NaCl:CTAB/Sarkosyl DNA extraction method the mycelium cultivation method of choice for cultivation of DNA and RNA extraction starting material changed. Initially liquid mycelium cultures were grown in 96 well plates for 10 days then harvested by pulling mycelium raft off of liquid surface and immediately freezing in liquid nitrogen for later use. Liquid cultures as opposed to agar cultures were initially preferred in order that the new *N. gardneri* transcriptome data would be more comparable to the a previously sequenced *N. gardneri* transcriptome. To combat low DNA yields, attempts were made to

increase DNA content of starting material by lyophilization, this did increase DNA yield but pigmentation was still problematic. The dark coloration of samples was likely due to a metabolic byproduct. There's a possibility that the bulk of the liquid weight was trapped liquid media, which is dark colored and potentially interferes with later steps in DNA extraction. Therefore a switch to agar cultures was made with mycelium grown above agar media separated by a dialysis membrane. This also increased DNA yield, but still did not solve pigmentation. Also due to the increased amount of cultures needed to be consumed while developing a DNA extraction method, the supply of Brazilian sugar cane molasses began to dwindle (these experiments were performed at Clemson University Genomics Center). It was at this point that malt extract produced for microbrewing was purchased, as it is known to taste like molasses and to foster the healthy and rapid growth of other fungal species in the beer making process. Culturing media was prepared with 5% malt extract in the same manner as molasses based media. *Panellus stipticus* had a strong effect by switching to malt based media with more rapid and healthy growth especially pronounced with the non-luminescent varieties of *P. stipticus*, additionally the luminescent varieties saw a marginal increase in light emission. The normally vigorous *L. edodes* culture grew equally great on either media. *Neonothopanus gardneri* did not react as kindly to the malt media, growth was retarded, live mycelium became pigmented, and growth was more flat on the media, nonetheless light emission still occurs albeit at a much lower level. Great effort was put into sensible and conservative consumption and then rationing of molasses in order to maintain normal growth of *N. gardneri*, but ultimately the Brazilian molasses supply ran out and a switch to culturing *N. gardneri* and *L. edodes* on malt media occurred which were used for DNA and RNA extraction.

3.2.6. RNA extraction

Mycelium samples for RNA studies were grown the same as mycelium for DNA studies on dialysis/agar malt media with the addition that frozen samples were collected at 8:00PM. RNA was simply extracted by using the Qiagen Plant RNeasy Kit following the standard plant extraction protocol with 100 mg of fresh, flash-frozen and powderized fungal mycelium. RNA samples were quantified and qualified with nanodrop and BioAnalyzer (RIN >8.0, Table 1). RNA samples were prepared four at a time, and ultimately the three best samples for each mycelium strain was sent as triplicates for transcriptome sequencing.

3.2.7. Genome sequencing

Ten micrograms of DNA for *N. gardneri*, *L. edodes*, *P. stipticus* luminescent, and *P. stipticus* non-luminescent was used to generate linear fragment and mate pair fragment libraries. Libraries were indexed by the standard procedure for the Illumina Nextera Library Kits and loaded onto a flow cell and sequenced on a single lane of an Illumina MiSeq 2x250 PE. Sequencing. Raw data processing of DNA and RNA sequencing was performed at the Clemson University Genomics Institute (CUGI) at Clemson University, Clemson, South Carolina.

Raw MiSeq reads from each sample were pre-processed to remove low quality and adapter sequences following k-mer filtration to 100x depth, when possible, to normalize the unique fraction of the data set for assembly. A de novo assembly was constructed from the linear fragment paired-end data set for construction of contigs. A secondary assembly was constructed using the longer-range mate pair paired-end dataset to scaffold and join contigs. The assembled genomes were then annotated using the MAKER software suite to predict coding sequences (Cantarel et al. 2008). The predicted coding sequences were then searched for functional signatures using translated alignments to the nonredundant protein database at NCBI and SwissProt, and domains profiled with InterProScan (Zdobnov and Apweiler 2001,

Goujon et al. 2010). Sequences were assigned GO terms using the Blast2GO software (Gotz et al. 2008).

3.2.8. Transcriptome sequencing

cDNA synthesis, fragmentation, library tagging and indexing was prepared according to the Illumina TruSeq Kit and run on a single lane of HiSeq 2x101bp PE. Raw HiSeq reads were pre-processed to remove low quality data, adapter barcode removal, and normalized with k-mer filtration, the global unigenes were assembled with the Trinity software package (Grabherr et al. 2011). The trinity output was aligned to the draft genomes with Bowtie2 (Langmead and Salzberg 2012). The individual transcriptome was assembled, transcript abundance estimated and differential expression calculated with Cufflinks and Cuffdiff2 (Trapnell et al. 2010). The final transcriptome was the sequence set with annotated genes and abundance (FPKM) values for each comparison. Like the genomes the predicted coding sequences are then searched for functional signatures using translated alignments to the nonredundant protein database at NCBI and SwissProt, and domains profiled with InterProScan (Zdobnov and Apweiler 2001, Goujon et al. 2010). Sequences were assigned GO terms using the Blast2GO software (Gotz et al. 2008).

3.2.9 Proteomics

The ultracentrifuge pellet of the crude extract contained luciferase separated from the hispidin 3-hydroxylase. The tris-glycine buffered solubilized pellet was loaded on an agarose gel and electrophoresed at 100 V, 30 mA. Initially horizontal 1 cm deep 2% agarose gels were used with tris glycine buffer. Later on thin vertical gels were used with the same gel composition. Gels were visualized by the application of horsetail extract or 3-hydroxyhispidin thin gel slices and visualized with the ImageQuant LAS 4000 mini gel imager with image sensitivity and pixel binning settings often set for maximum light detection for weak emitting source. Gel segments

that were observed glowing were further dissected to remove non-luminescing gel and frozen for later trypsin digestion and mass spectrometry.

The ultracentrifuged pellet of the crude extract contained luciferase was also passed through a HiTrap DEAE ion exchange column (GE) using a GE AKTA FPLC. The proteins were first passed through a syringe filter to remove large particles prior to loading onto the column. The binding buffer was tris buffer (25 mM, pH 7.0), the elution buffer had additionally 1M NaCl. Protein extract was loaded onto the column and washed with 3 mL of buffer prior to elution along a gradient between loading and elution buffer over 5 mL. Fractions were collected in 500 μ L or less volumes. 100 μ L of fraction were used for bioluminescence assay with equesitumprone. Samples with maximum luminescence were frozen for later trypsin digestion and mass spectrometry.

3.2.10 Bioinformatics

The genome and transcriptome assembly sequence files, MAKER gene annotation files, transcript abundance values and identified peptide fragments from mass spectrometry were organized and parsed with custom python scripts relying heavily on biopython (Cock et al. 2009) to handle sequence data. In addition the genomes of other agaricales species were downloaded from NCBI and JGI (Grigoriev et al. 2012) servers. Gene sequences were re-annotated with an additional run of InterProScan, and the KEGG online annotator (Moriya et al. 2007). In addition to the new transcriptome sequences described above, a transcriptome set of *N. gardneri* was provided by Anderson Oliveira, IO-USP and Jay Dunlap, Dartmouth University Medical School. Additional fungal cytochrome P450 seed sequences were downloaded from the Fungal Cytochrome P450 Database (Park et al. 2008). The transcriptome sequences were mapped to genome scaffold assemblies using BLAT (Blast Like Alignment Tool, Kent 2002) and cross referenced with neighboring and overlapping maker predicted genes. MAKER cds sequences

and translated transcriptome sequences were passed through additional EMBOSS suite annotation software (Rice et al. 2000) for structural information (molecular mass, transmembrane domains, 2D structure). Seed sequences were BLASTed (Altschul et al. 1990) against the genome and transcriptomes in various ways to identify homologs in addition to functional annotation word analysis. CDS sequences of identified genes of interest often were compared to similar genes using Clustal Omega (Sievers et al. 2011) and phylogenetic trees created with MEGA or Phylip (Kumar et al. 2016, Felsenstein 1989) and visualized with biopython calls or SeaView (Gouy et al. 2010).

4. Results

4.1. Ecology

4.1.1. Bioluminescent Mushrooms in the forest

Figure 4: Bioluminescent mushrooms of the Atlantic Rainforest. A, left, *M. luxaeterna*, center, *G. viridilucens*, right green LED light 5V, 1M Ω resistor, dark photograph 20 s, ISO 12800, f 2.0. B, *Mycena lucentipes*, 5 min, ISO 800, f 2.0. C, *Mycena globulispora*, 5 min ISO 800, f 2.0. D, Uncharacterized *Mycena* species, US penny reference, 30 s ISO 1600, f 2.0.

Beginning in 2012, numerous forest field excursions began for the search of bioluminescent mushrooms. In the Atlantic Rainforest of São Paulo state numerous species were encountered. The most commonly encountered species was *Mycena luxaeterna*, a small mushroom with 3-5 cm luminescent stipe and non luminescent pilus (Fig. 4A). This species was encountered in great numbers at the private field station of IPBio, but was also frequently encountered at the Núcleo Santana entrance in PETAR. Both locations are along the same river, rio Betari. *Mycena luxaeterna* exclusively found on decaying plant matter on the forest floor, typically found on dead leaves and twigs of ingá, juçara palms, and *Melastomataceae* plants, identified by myself and IPBio staff, Henrique Domingos. The next most commonly encountered species in the Atlantic Rainforest was *Gerronema viridilucens*, a reddish mushroom with a vase shaped luminous pilus and dark non-luminous stipe (Fig. 4A). This species was encountered at IPBio and Núcleo Santana, but also found at several other locations within the state park, Laje Branca and Núcleo Caboclos. *Gerronema viridilucens* was found growing on free twigs lying on the forest floor, dead bark, the base of living and dead trees and exposed rotting roots. The next most encountered mushrooms species in the Atlantic Rainforest was *Mycena lucentipes*, a small 3 to 5-cm mushroom that also luminescences from the stipe (Fig. 4B). This species was reported to be encountered more commonly the previous decade, nowadays it was only encountered a handful of times in Núcleo Santana and Laje Branca. This species is commonly found on plant litter scattered on the forest floor. Fallen palm fronds in the Atlantic Rainforest are often found with a coating of luminescent mycelium, associated with the mycelium are the small, 2 mm wide non-luminescent fruiting bodies of *Resinomyces petarensis*. Beginning in 2014, *Mycena globulispora* fruiting bodies started to be encountered more frequently (Fig. 4C). This small mushroom with 2 cm stipes and 2 cm pilus, is exclusively found above the forest floor on dead tree branches several meters off the ground. It

has been encountered at IPBio, Laje Branca, and Núcleo Caboclos. It has been encountered in warmer months and it appears to be more prolific in June and July, when cooler nights are more frequent. In addition to the aforementioned species, which were found with some frequency, several other luminescent mushroom species were found on occasion. *Mycena asterina* was found again one time since 2012 in Núcleo Caboclos, *Mycena oculisnympphae* a small 1 cm wide mushroom found on the trunks of dead trees in IPBio and Núcleo Santana. A further unknown species was photographed a small (1-2 mm wide) mushroom found on tree trunks (Fig. 4D).

Figure 5: Top, *Neonothopanus gardneri* mycelium growing within the base of a senescing babaçu palm frond. Bottom, *N. gardneri* mushrooms, note the Lycosidae spider above the right mushroom cluster.

Excursions to Altos, PI were undertaken to encounter *Neonothopanus gardneri* (Fig. 5) The entire yellow fruiting body of *N. gardneri* is brightly luminescent, making the mushroom easy to locate from a distance with mature fruiting bodies often greater than 10 cm in height and

diameter. The pilus is vase shaped with no clear distinction between the pilus and the stipe. *N. gardneri* is widely distributed in the area, it has been found in stands of babaçu palms (*Attalea speciosa*) searched near streams and creeks. On optimal nights and in fresh unharvested stands it is not uncommon to encounter over 50 mushrooms. The ground mycelium of this species is also quite prolific and can be readily seen during heavy rains that hydrate the soil, resulting in a weak glow from the forest floor that can be made brighter by disrupting the soil. The glow of ground mycelium can radiate several meters from a babaçu tree with a luminous mushroom. The mycelium of *N. gardneri* can also be observed by breaking open palm fronds that had a glowing mushroom growing on it, once broken open and with the addition of water mycelium can be seen growing throughout the interior of the palm frond (Fig. 5).

Figure 6: Top, *Panellus stipticus* found in Caesars Head state park in South Carolina, 25 s, ISO 1600, f 1.8. Bottom, *Armillaria* sp. found in the Clemson University experimental forest, 5 min, ISO 3200, f 2.0.

Additionally the luminescent mushroom, *Panellus stipticus* was found in the southern Appalachian mountains in the South Carolina state park, Caesars Head. *Panellus stipticus* fruiting bodies have short stripes and a 2-5 cm wide elliptical caps and grow in clusters (Fig. 6). Unlike the species encountered in the Atlantic Rainforest, which were all found in close proximity to streams or rivers, *Panellus stipticus* was found along mountain ridges and not found in the stream basins located downhill. *Panellus stipticus* fruiting bodies were found growing on various substrates, twigs on the ground, exposed and rotting roots, and on the trunks of dead trees at about eye level. A patch of honey mushrooms (*Armillaria* sp.) was encountered in the Clemson University Experimental Forest, in Clemson South Carolina. The mycelium of several American and European *Armillaria* species have been known to be brightly luminescent, but the fruiting bodies have not been observed to be as well. Nonetheless, this patch of mushrooms was first sighted in the dark by weak light coming from the fruiting bodies. Long exposure photography also detected light from the fruiting bodies when photographed in the field (Fig 6), but when these mushrooms were collected and photographed several hours later in a dark room no light could be detected.

4.1.2. Arthropods captured in glowing mushroom facsimiles

A total of 1,186 arthropods were identified from 273 glue traps from the ecology experiments in the Atlantic Rainforest in three sets between March 2012 and July 2013. The first set of collections was performed over nine nights between March 28th and April 25th of 2012, when 85 control traps and 87 light traps were used. The second set was conducted over five nights between February 24th and March 3rd 2013, when 26 control traps and 25 light traps were used. The third set occurred over five nights between July 5th and July 10th of 2013, 25 control traps and 25 light traps were used. The *M. luxaeterna*-like traps were placed on the forest floor in areas where the mushrooms naturally occur.

Table 1: Arthropod prevalence on glue traps from the Atlantic rainforest. Orders statistically significant (binomial p-value < .05) are marked with an asterisk (*)

	LED Traps	Control Traps	<i>P-value</i>
Acari	4	4	0.6407
Araneae*	73	49	0.0167
Archaeognatha*	6	0	0.0153
Blattodea	10	6	0.2228
Coleoptera*	79	50	0.0060
Collembola*	45	23	0.0047
Diptera*	293	217	0.0001
Hemiptera*	101	46	0.0000
Hymenoptera	34	33	0.4880
Isopoda	0	2	0.2518
Isoptera	3	6	0.2575
Neuroptera*	24	1	0.0000
Opiliones	1	0	0.4982
Orthoptera*	59	17	0.0000
Total Arthropods	732	454	0.0000

Sixteen different orders of arthropods were identified (Table 1 and 3) of which the Acari, Araneae, Coleoptera, Collembola, Diptera, Hemiptera, Neuroptera and Orthoptera were found in numbers significantly greater on the luminescent glue traps. In particular, Diptera and Hemiptera were found in statistically significant greater numbers from each set of experiments. While hymenopterans were captured in great numbers, they were found in roughly equal numbers on light and dark traps. There was a sharp decline in the number of coleopterans found on traps in the sets from 2013 compared to 2012, the noticeable difference between these sets is mainly due to the presence or absence of a single species of an staphylinid beetle. The arthropods captured on the traps are likely only a partial representation of the total arthropods that interact

with the mushrooms as only smaller arthropods (< 1.0 cm) were captured by the glue, larger insects were not typically found on the traps after collection. On several occasions during the collection of the traps from the field, it was observed that larger arthropods, typically Araneae (spiders), were stuck on the traps but would quickly, within 5 min, free themselves.

Table 2: Arthropod prevalence on glue traps from Piauí's transitional palm forest. Orders statistically significant (binomial p-value < .05) are marked with an asterisk (*)

	LED Traps	Control Traps	P-value
Araneae	3	2	0.5000
Blattodea	1	0	0.5000
Coleoptera*	6	0	0.0156
Diptera*	17	4	0.0036
Hemiptera	4	0	0.0625
Hymenoptera	7	2	0.0898
Isoptera	1	3	0.3125
Lepidoptera	1	0	0.5000
Orthoptera	1	0	0.5000
Plecoptera	1	0	0.5000
Total Arthropods	42	11	0.0000

Glue trap experiments were performed in Piauí between March 7th and March 18th 2013, 19 light glue traps and 19 control glue traps were recorded on 5 nights. Overall, there was much less biodiversity of arthropods, plants, and fungi in the Babaçu Forest test area. Only 10 orders of arthropods were observed in the glue traps and from direct collection from mushrooms (Table 2 and 3), mainly spiders, cockroaches, click beetles, ants, termites, and flies. On average only 1.4 arthropods were caught per trap compared to 4.3 per trap in the Atlantic Rainforest. Only two orders, Coleoptera and Diptera, were statistically in greater numbers on the light traps

compared to controls. Like the Atlantic Rainforest traps, only smaller ($< 1.0\text{cm}$) arthropods were caught in the glue, but much larger arthropods were caught from direct collection from the mushrooms. When walking around the field site and collecting mushrooms it was quite common to see large spiders in close vicinity to the mushrooms. Arthropods were removed from mushrooms after collection, sometimes after more than 30 min. These arthropods included Blattodea, Dermaptera, Hemiptera, Hymenoptera, and Lepidoptera. The collected Dermaptera were often found burrowed deep into the stipe of older mushrooms.

The most diverse orders were Coleoptera (beetles), Diptera (flies), Hemiptera (true bugs) and Hymenoptera (Wasps and Ants). These four orders were further identified to the family level (and to the species level when possible) with the help of prof. Silvio Nihei, IB-USP (Table 5). Thirteen Coleopteran families were collected from glue traps. The total number and diversity of Coleoptera found on traps was high, yet the difference between LED and control traps was barely significant, further separation of the order did not find any families to have strong statistical significance for attraction or repulsion to the green light, although the family Staphylinidae was borderline significant. There were four Dipteran families found to be statistically influenced by the green light (Table 4). Three Brachycera families were found in statistically relevant numbers Atlantic Rainforest traps, two Muscidae and Phoridae were attracted to light, whereas the Mesembrinellinae were repelled (Table 5). The Nematoceran family Sciaridae was attracted to green light in Piauí (Table 5). The Cicadellida family of Hemiptera was strongly attracted to green light (Table 4 and 5). Like Coleoptera, Hymenoptera were found in large numbers with a large diversity of individuals with eight families identified. However, the order as a whole did not have trap preference, ants from the family Formicidae were strongly repelled by the green light traps (Table 4 and 5). In addition to the other previously described orders, six families from these four orders were affected by the green light.

Table 3 Results of the 16 arthropod orders found on the glue traps in the Atlantic Rainforest. Orange values are total arthropod counts found on luminescent traps and control traps. Yellow values are counts from different trips by year on March or on July. Pink values are the binomial test statistics of LED vs. control, significant values < 0.05 and are highlighted in a darker shade. Green values are the binomial p-values for the comparison of total March and July arthropod counts.

Label	PETAR Control Total	2012 Con.	2013 Con.	2013 July Con.	PETAR Light Total	2012 Light	2013 Light	2013 July Light	PETAR Total Binomial Test	PETAR 2012 Binomial Test	PETAR 2013 Binomial Test	PETAR 2013 July Binomial Test	PETAR Mar. v. Jul. Binomial Test	Label
Acari	4	1	3	0	4	1	1	2	0.64	0.76	0.30	0.25	0.82	Acari
Araneae	49	30	12	7	73	33	10	30	0.02	0.37	0.38	0.00	0.00	Araneae
Archaeognatha	0	0	0	0	6	4	1	1	0.02	0.06	0.51	0.50	0.45	Archaeognatha
Blattodea	6	4	0	2	10	7	2	1	0.22	0.26	0.26	0.50	0.24	Blattodea
Coleoptera	50	46	4	0	79	65	9	5	0.01	0.03	0.15	0.03	0.00	Coleoptera
Collembola	23	11	3	9	45	29	2	14	0.00	0.00	0.48	0.20	0.00	Collembola
Diptera	217	200	10	7	293	227	43	23	0.00	0.01	0.00	0.00	0.00	Diptera
Hemiptera	46	35	9	2	101	70	22	9	0.00	0.00	0.02	0.03	0.00	Hemiptera
Hymenoptera	33	26	5	2	34	22	7	5	0.49	0.36	0.41	0.23	0.01	Hymenoptera
Isopoda	2	2	0	0	0	0	0	0	0.25	0.26	1.00	1.00	1.00	Isopoda
Isoptera	6	6	0	0	3	3	0	0	0.26	0.27	1.00	1.00	0.84	Isoptera
Neuroptera	1	1	0	0	24	16	8	0	0.00	0.00	0.00	1.00	0.04	Neuroptera
Opiliones	0	0	0	0	1	1	0	0	0.50	0.49	1.00	1.00	0.97	Opiliones
Orthoptera	17	14	2	1	59	55	3	1	0.00	0.00	0.52	0.75	0.00	Orthoptera
Num Traps	136	85	26	25	137	87	25	25						
Sum	454	376	48	30	732	533	108	91						

Table 4: Family prevalence of the orders Coleoptera, Diptera, Hemiptera and Hymenoptera. Groups with significant difference (p-value < 0.05) between LED and control traps were attracted (green), repelled (red), or mixed (yellow). Groups borderline significance are italicized. Total number of families are in parentheses, a count of families with significant response is the second number within parentheses.

	PETAR control	PETAR light	P-value	Piauí control	Piauí light	P-value	Piauí direct
Coleoptera (13)	51	70	0.06	2	4	0.34	19
Bostrichidae (1)	1	0	0.50				
Cucujiformia (4)	4	7	0.28	2	0	0.25	2
Elateriformia (2)	1	1	0.75				
Scarabaeidae (1)	0	1	0.50				
Staphylinoidea (4)	37	51	0.09	0	4	0.06	17
Carabidae (1)	8	10	0.41				
Diptera (14:4)	189	254	0.00	4	14	0.02	0
Brachycera (9)	174	239	0.00	4	1	0.19	0
Nematocera (5)	15	24	0.10	0	16	0.00	0
Hemiptera (5:1)	41	92	0.00	0	4	0.06	1
Cicadomorpha (2)	37	87	0.00	0	3	0.13	1
Fulgoromorpha (3)	4	5	0.50	0	1	0.50	0
Hymenoptera (8:1)	24	22	0.68	2	3	0.50	5
Wasps (7)	12	19	0.15	2	1	0.50	1
Ants (7)	12	3	0.02	0	2	0.25	4

Table 5: Specific families showing green light positive phototaxis (green) or repulsion (red) in the Atlantic Rainforest (PETAR) and Piauí.

PETAR	Piauí
Diptera: Brachycera	Nematocera
Mesembrinellinae	Sciaridae
Muscidae, <i>Musca domestica</i>	
Phoridae	
Hemiptera: Cicadellida	
Cicadellida	
Hymenoptera: Vespoidea	
Formicidae	

4.1.3. Arthropod activity on and around luminous mushrooms

Following the glue trap studies, an infrared video observation study began. Initially in 2013, preliminary IR video documentation was performed in the Atlantic Rainforest and Piau alongside glue traps, the infrared camera used was only able to capture 3 h of video effectively per night due to limited battery capacity. The preliminary IR video data painted a very different picture of the interaction of arthropods and the mushrooms than the glue traps. In the Atlantic Rainforest preliminary IR video three main groups of arthropods were observed with interesting dynamics between them: Blattodea (cockroaches), Opiliones (harvestmen), and Hymenoptera (ants). Similar to the Atlantic Rainforest preliminary IR video, *N. gardneri* mushrooms were also filmed at night, and three 1 hour segments were captured. There were four groups of arthropods observed on the preliminary Piau IR video, Blattodea (cockroaches), Araneae (spiders), Hymenoptera (ants) and to a lesser extent Coleoptera (beetles). With the intriguing preliminary results a large scale infrared time lapse photography study of the nocturnal niche surrounding luminescent mushrooms was initiated using four modified cameras to capture the entire night of activity.

4.1.3.1. IR infrared Atlantic Rainforest

The massive collection of infrared time lapse footage from the Atlantic Rainforest was undertaken from late February 2014 until late March 2014, when mushrooms were commonly found and consistent with the previous glue trap studies. A number of 1,346 arthropods and various other animal events were observed from 27 videos of *M. luxaeterna* and 3 control videos from the Atlantic Rainforest (Table 6). Each video was about 8 h long and covered from just after sunset until sunrise.

The most frequently observed arthropod order around the *M. luxaeterna* mushroom was Hymenoptera. Almost all of these sightings were ants although a few wasps or other winged

hymenoptera were observed. In both the IR and control videos the ants seemed disinterested in the actual mushroom and were seen wandering through the observable view. Nonetheless, there were statistically greater ants seen in the IR videos of *M. luxaeterna* mushrooms than controls views of ground with no mushroom. While ants seemed uninterested in the mushrooms they did seem to interact and bother other insects they encountered, often startling encountered cockroaches and opiliones, which would subsequently flee the area.

Table 6: Arthropod and other animal Infrared event counts from the Atlantic Rainforest. 27 Infrared video sets were annotated compared to three control video sets, values with a binomial test statistic value below 0.05 were considered significant.

<u>Event</u>	<u>total</u>	<u>IR(27)</u>	<u>control(3)</u>	<u>Binom. value</u>
Hymenoptera	471	441	30	0.00
Blattodea	205	198	7	0.00
Opilione	176	168	8	0.01
Small ground	121	88	33	1.00
Worm	70	65	5	0.29
Flying	51	40	11	1.00
Spider	43	41	2	0.18
Orthoptera	24	22	2	0.56
Centipede	14	13	1	0.58
Snail	14	14	0	0.23
Millipede	7	7	0	0.48
Slug	7	4	3	1.00
Archaeognatha	3	3	0	0.73
Swarm	2	2	0	0.81
Bird	1	0	1	0.10
Coleoptera	1	1	0	0.90
Earwig	1	0	1	0.10

The second and third most common arthropods observed near *M. luxaeterna* were forest cockroaches (Blattodea) and harvestmen (Opiliones). Cockroaches were the most frequent arthropod observed eating the *M. luxaeterna* mushroom. If a given cockroach was left undisturbed it would continue to feast upon the mushroom cap until it was gone, at which point

the cockroach would exit the observable view. Most times, only the pileus was consumed by the cockroaches, leaving the luminescent stipe, but on 3 occasions the cockroach consumed both pileus and stipe. Cockroaches were also observed several times laying eggs in camera view, this was observed in both *M. luxaeterna* and control videos, the cockroaches did not seem to pay much attention to this act as they would often lay the eggs in mid stride. Opiliones (harvestmen) were also frequent eaters of the *M. luxaeterna* mushrooms. Like the cockroaches, they only ate the mushroom pileus leaving the luminescent stipes untouched. Cockroaches and Opiliones would often fight over a luminescent mushroom. Opiliones also fought amongst themselves over feeding rights with often the larger individual typically winning, the losing Opilione would often remain in the general vicinity until the larger Opilione or cockroach left the mushroom.

Small ground spiders, mainly from the family Gnaphosidae, were also frequently observed in the area of *M. luxaeterna*, their behavior seemed more similar to ants, namely they were frequently seen just passing through the viewing area not seeming to pay much attention to the luminescent mushrooms. Slightly larger spiders were seen in the videos, likely of the family Lycosidae. On most occasions, they would stop for a longer period of time, up to 30 min, often with their bodies pointed in the direction of the mushrooms, the movement of other insects in the vicinity would trigger movement of the Lycosidae but no attacks were observed in the Atlantic Rainforest.

There was about 1 hour of video time when crickets were observed in the viewing area. Crickets (Orthoptera) were also frequently observed feeding on the *M. luxaeterna* mushrooms, although they were not nearly as commonly observed eating as cockroaches and opiliones. Like the other fungivores, the crickets did not eat the mushrooms all times observed, but they were often just passing through the viewing area.

Moreover, several other groups of animals were observed on occasion in the mushroom viewing area at night. Worms were quite frequently seen following rain storms, often when a worm was observed it was seen in the viewing area for quite some time squirming around the general area. Millipedes also made a few appearances, passing through the viewing area seeming to pay no attention to mushrooms. On one occasion, a millipede interrupted a feeding opilone and caused it to leave the area. Centipedes were also observed in limited numbers in the Atlantic Rainforest, but they also did not seem to notice the bioluminescent mushrooms. Archaeognatha were observed three times around luminescent mushrooms but only remained in view for a few seconds. Lastly, several slugs and snails were observed, most were seen just before and after sunrise, but on no occasion was a slug seen eating a mushroom.

While resolution of the modified GoPro cameras was improved compared to the preliminary IR camera, smaller arthropods and details of the larger ones were still below the minimum resolution resulting in almost all of the flying insects and many of the small ground dwelling insects being detected, but unable to be identified to any great depth. The behavior of these large groups was diverse, with some insects just passing through the video area and others spending quite some time near the mushrooms. On no occasion were the smaller insects or small flying insects obviously eating the mushrooms, but this possibility cannot be discarded. On two occasions near *M. luxaeterna*, swarms of small ground insects were seen in the area with numerous small insects moving together as a group, some of the insects crawled on the mushroom, but overall were crawling on everything in their path.

4.1.3.2. IR Infrared transitional palm forest

Infrared video recorded with IR modified GoPros occurred in February 2014 and March 2015. A number of 2,517 different animal events were observed, ranging from tiny unidentifiable insects to domestic pigs among 4.5 million timelapse images. Mainly, larger insects were

observed, the most common being Blattodea (cockroaches), Hymenoptera (ants and wasps), spiders, beetles and centipedes (Table 7). Cockroaches were frequently seen eating the bioluminescent mushrooms (Fig. 7). Additionally, pigs ate entire mushrooms and earwigs burrowed into the mushrooms. Cockroaches were observed quite frequently with over 40 h of footage of them, in the control videos but they did not loiter in the viewing area nearly as long as when a bioluminescent mushroom was present. Cockroaches also appear to be most active around bioluminescent mushrooms from sunset until midnight in Piauí. After sunrise, cockroaches were not observed as frequently.

Figure 7: The rapid mycophagy of a *N. gardneri* mushroom, green outline, by a single cockroach, red outline, in a ten minute period.

The main hymenopterans observed in the infrared videos were ants. Typically an ant would enter frame, wander through, and then exit. The frequency of ants passing through the camera view was fairly constant when ants were present. Often, multiple ants would be in the viewing area at once, and surely a single ant would make multiple passes through the viewing area. Nevertheless, the ants did not seem to be paying any attention to the mushroom. Except one night, when the ants were quite present, with one of them almost always in view. About an hour before sunrise *ca.* 5 ants centered on the mushroom in a frenzy and eventually began pulling at the mushroom with their mandibles. This unique observed behavior ended with a

sunrise rainstorm. Aside from this single frenzied attack, the ants had never seemed to notice or pay much attention to bioluminescent mushrooms.

Table 7: Arthropod and other animal Infrared event counts from the Coconut Forest of Piauí in 2014 and 2015. Thirty three Infrared video sets were annotated compared to 15 control video sets. Values with a binomial test statistic value below 0.05 were considered significant.

<u>event</u>	<u>total</u>	<u>IR</u>	<u>control</u>	<u>Binom. value</u>
Hymenoptera	1205	1070	135	0.00
Blattodea	259	235	24	0.00
Earwig	144	142	2	0.00
Spider	135	86	49	0.92
Flying	117	90	27	0.04
Coleoptera	113	110	3	0.00
Small ground	91	78	13	0.00
Centipede	52	46	6	0.00
Opilone	44	39	5	0.00
Worm	19	13	6	0.63
UNKNOWN	16	16	0	0.00
Snail	12	7	5	0.87
Pig	8	3	5	0.99
Orthoptera	7	7	0	0.07
Scorpion	5	1	4	0.04
Diptera	4	4	0	0.23
Neuroptera	4	4	0	0.23
Rat	4	3	1	0.63
Stick Bug	4	2	2	0.91
Gastropod	3	1	2	0.23
Lizard	3	0	3	0.03
Snake	2	1	1	0.90
Mouse	1	0	1	0.31

Spiders were also commonly observed in the areas around the bioluminescent mushrooms. The main spider seen in the infrared video is likely to be a Brazilian wolf spider of the family Lycosidae. These spiders were also seen in the control videos. Most observations of the spiders are of them passing by the mushroom or stopping for only a few minutes, but often

the spiders stopped in view of the mushroom and stayed for up to an hour. On one occasion, when a cockroach approached to the mushroom, the awaiting spiders attacked the cockroach. In the control time lapse videos the spiders also stopped and appeared to be waiting for a prey, but they did not stay at this position as long as they do in the presence of a mushroom.

Interestingly, the most commonly observed beetle in the IR video were bioluminescent click beetles, whose green glow was clearly detected. Click beetles would fly or walk onto the bioluminescent mushrooms, they would crawl around on the mushroom for a few seconds and then fly away, with the whole event lasting less than a minute. Some beetles would return to the mushroom only seconds after flying away, multiple times, and often the beetles would arrive in pairs with both beetles crawling around on the mushroom together, and then flying away. The click beetles do not seem to feed on the mushroom, nor do they seem to be laying eggs, they appear to just be arriving, walking around on the mushroom and flying off.

In 2014 footage, earwigs were commonly seen with *N. gardneri*. One or two earwigs would crawl around and on top of mushrooms for the whole duration of the night when observed. There was no clear sign of them feeding upon the mushrooms, but they persisted at a given mushroom and would attack other intruding insects scaring them off. In addition to IR footage of the earwigs, several were found in collected mushrooms hours after harvest, having burrowed into the interior mycelium of the mushroom stipe, this is consistent with IR video.

Other arthropods were also observed in the IR videos, but not nearly in the same numbers, larger tarantulas, Opiliones, neuroptera, stick-bugs, pseudo-scorpions, scorpions, and centipedes. There were many smaller insects detected but not identifiable to any degree beyond detection, these insects were grouped as small ground insects or small flying insects, the numbers of both groups were high and seemed to be greater around bioluminescent mushrooms. On multiple instances swarms of small ground insects were seen crawling on the

large bioluminescent mushrooms. Due to image resolution limitations it was impossible to identify these swarming insects, but they were clearly seen interacting with the mushrooms for several minutes before exiting the observable area. Arthropods were not the only phylum of animal observed in the Piauí IR footage, worms were often seen, and often seen around the bioluminescent mushrooms. Snails were commonly observed immediately after sunrise, but not during the night. On a few different occasions snakes were observed, and also field mice, rats and birds. On multiple occasions domestic pigs were seen in the videos, unlike the other non-insect animals, pigs also seemed to see the mushrooms and eat them. This typically occurred about an hour before astronomical sunrise. In addition to the small ground and flying insects, which were detected insects but unidentifiable, there were a number of larger insects in Piauí that were also not able to be categorized. The ones most commonly seen were dark > 5 cm oval shaped insects, crawling up and down the palm frond with a bioluminescent mushroom. These unknown insects in some ways resembled cockroaches, but their movement pattern did not match to the cockroaches one. Moreover, other aspects of their visual appearance did not match with cockroaches as well.

In the second year of Piauí Infrared observations more control footage was gathered, by observing juvenile babaçu palms without mushrooms or other mushrooms found in the area that were not bioluminescent (Table 8). Hymenoptera were seen quite frequently in both bioluminescent mushroom and control videos, but they were found in statistically greater numbers around luminescent mushrooms. Even though, they did not seem to be greatly interested in the mushrooms themselves with the exception of the aforementioned single occurrence of the frenzied attack of a *N. gardneri* mushroom by ants. Surprisingly, the number of spider observations was similar in both luminescent mushroom and control time lapse sets, although the average amount of time spent near the mushrooms was greater for luminescent

mushrooms. Coleoptera, mainly click beetles, were seen in statistically greater numbers in IR video with *N. garneri* than controls.

Table 8: Arthropod and other animal Infrared event counts from Coconut Forest of Piauí in 2015 alone, when control videos were consistently generated. Thirteen Infrared video sets were annotated compared to 14 control video sets. Values with a binomial test statistic value below 0.05 were considered significant.

Event	total	IR	con	Binom. value
Hymenoptera	413	278	135	0.00
Spider	98	50	48	0.38
Blattodea	95	71	24	0.00
Flying	61	34	27	0.18
Small ground	30	19	11	0.08
Coleoptera	27	24	3	0.00
Opilone	26	21	5	0.00
Centipede	16	10	6	0.20
Worm	10	4	6	0.42
Snail	9	4	5	0.55
Pig	7	2	5	0.26
UNKNOWN	6	6	0	0.01
Scorpion	5	1	4	0.21
Gastropod	3	1	2	0.53
Lizard	3	0	3	0.14
Orthoptera	3	3	0	0.12
Rat	3	2	1	0.49
Earwig	2	0	2	0.27
Snake	2	1	1	0.77
Stick bug	2	0	2	0.27
Mouse	1	0	1	0.52

4.2. Enzymology

4.2.1. Comparison of light intensity of luminous cultures

The light emission of the staple cultures used throughout the study has been observed extensively in a dark room, The mycelium of *N. gardneri* cultures grown on media containing 5% molasses with 0.1% yeast extract are the brightest, along with the luminescent varieties of

Panellus stipticus, which are comparatively bright. *G. viridilucens* and *O. olearius* cultures are noticeably luminescent when viewed in a dark room but are significantly dimmer than luminescent *N. gardneri* and *P. stipticus* strains. This visual observation is supported by measurement with a Tecan luminometer (Fig. 8) and other photosensitive devices.

Figure 8. Light emission of luminous fungal mycelium cultures routinely used for experiments with a Tecan Infinite multiwell plate reader with a custom 40mm petri dish holder. Cells for each petri dish were averaged, measurements were performed in triplicate for each culture.

Different culturing strategies were tested and used at times for various tasks. Cultures grown on dialysis membranes above agar yielded more luciferase-rich extracts, likely due to the significant decrease in wet mass from the starting material. The actual choice of sugar source also seemed to make quite a difference. *N. gardneri* grew optimally on blackstrap molasses, whereas it was significantly dimmer and had less healthy growth in malt extract. *P. stipticus* light output was minimally enhanced on malt extract based agars, but other growth metrics were improved such as growth rate, and luminescence uniformity. *P. stipticus* and *N. gardneri* are also

capable of growing and glowing on potato dextrose agar and rice flour. They struggle to glow on tryptone and lactone based agars with added dextrose. Additionally the selection of blackstrap molasses is important, the best blackstrap molasses is truly organic and raw molasses. All tested store bought brands of molasses are able to sustain growth of *N. gardneri* but are only minimally luminescent (Fig. 9). The ratio of store bought to raw molasses was varied by 10% increments and light emission was only observed to be strong when the ratio was above 8:2 raw:store molasses.

Figure 9. *Neonothopanus gardneri* grown on store bought vs organic molasses. The petri dish pair to the far left were grown on pure organic molasses, next are 4:1 organic to store bought, 3:2, 2:3, 1:4, and to the far right were grown on pure store bought molasses.

4.2.2 Experiments with the crude extraction of luciferase from *Neonothopanus gardneri*

The crude protein aqueous extract of *N. gardneri* catalyzes the cell free light emission with the addition of the fungal luciferin, 3-hydroxyhispidin. Light can also be obtained by the addition of hispidin and NAD(P)H to the crude extract to provide hispidin to the hydroxylase yielding 3-hydroxyhispidin whose light emission is then catalyzed by the luciferase (Fig. 10A). Light emission from pure sources of hispidin, 3-hydroxybisnoryangonin and 3-hydroxyhispidin yield much more intense light than the hot-extracts from fungal sources, which were the only known source of luciferin-rich samples prior to the identification of hispidin as luciferin precursor in 2015. Weak but above background noise levels of light were detected when NADPH was

added to the crude extract incubated with caffeic acid, ATP, and malonyl-CoA, suggesting that caffeic acid can be converted to hispidin via the activities of 4CL and the polyketide synthase responsible for hispidin biosynthesis (Fig. 10B).

Table 9: Initial screening of plant extracts with fungal luciferase for bioluminescence activity. Light emission is the maximum value during the observed time (> 10 min).

Plant	Tissue	Light emission (RLU)	Notes
<i>Equisetum hyemale</i>	Strobili	>20M	
<i>Equisetum hyemale</i>	Root	5M	
<i>Casearia sylvestris</i>	Leaf	<1K	
<i>Pistia stratiotes</i>	Leaf	400K	Max light at 30min
<i>Arabidopsis thaliana</i>	Leaf	3k	
<i>Plectranthus amboinicus</i>	Leaf	2K	Required NADPH
<i>Capsicum</i>	Leaf	2k	
<i>Dunaliella salina</i>	Algae	<500	
<i>Citrus</i> (lime)	Leaf	<500	
<i>Citrus</i> (lime)	Exocarp	3K	
<i>Citrus</i> (lime)	Pericarp	<500	
<i>Citrus</i> (lime)	Juice sac	<500	
<i>Piper tuberculatum</i>	Leaf	10K	
<i>Piper nigrum</i>	Leaf	2K	
<i>Piper auritum</i>	Leaf	6K	
<i>Piper aduncum</i>	Leaf	2K	
<i>Piper richardfolium</i>	Leaf	2K	
<i>Saccharum</i>	Leaf	<500	
<i>Taxus baccata</i>	Leaf	<500	
<i>Juniperus</i>	Leaf	<500	

A.

B.

Figure 10. A. Solubilized luciferase pellet with 3-hydroxybisanryangonin (blue) and horsetail equisetumprone (red). B. Light emission triggered by the addition of NADPH (50 μ L, 1 mg/mL) to the crude fungal extract (60 μ L 1:2 diluted in extraction buffer) incubated for 1h (red) with caffeic acid (10 μ L, 40 mM), malonyl-CoA (5 μ L, 20 mM), ATP (10 μ L, 10 mM), - control with no addition of NADPH (blue).

In addition to fungal and synthetic sources of bioluminescent active compounds, several plant species proved to be ready sources of luciferin-like compounds. The brightest of those tested were of the genus *Equisetum*, horsetails (Fig. 10A). The second brightest plant extract tested was derived from the the leaves of *Pistia stratiotes* (water lettuce, alface d'agua), which displayed extremely odd in vitro light emission kinetics, yielding maximum light emission of 400,000 RLU after 30 min and over 2 hours until the light was extinguished. Several other plants yielded low but detectable light emission, guacatonga (*Casearia sylvestris*), several species from the *Piper* genus, *Arabidopsis thaliana*, *Citrus* sp., *Capsicum*, (Table 9). Given the important role of *Arabidopsis* in plant molecular research, it was attempted to concentrate the methanolic extract of *Arabidopsis* leaves in order to obtain greater light emission. With this in mind, 10 g of leaves were extracted with 25 mL of ethanol and roto-evaporated, the resulting residue was suspended in 1mL of ethanol. This solution has a deep red color contrasting with

the starting solution that was green. When the red extract of *Arabidopsis* was tested with fungal luciferase less than 500 RLU of light was detected, typical value of no light emission. Additionally, this red solution was combined with horsetail extract and fungal luciferase extract and also no light was observed. The addition of sodium hydroxide to the red extract of *Arabidopsis* changed the color of the solution first to blue than to green, suggesting that the inhibitory molecules might be anthocyanins. All tested pines, green algae (*Dunaliella salina*), sugar cane sprouts, and *Acacia* leaves did not have any detectable light emission.

4.2.3. Luciferase partial purification

Ultracentrifugation of the crude extract to separate the luciferase from the hydroxylase was generally successful. Ultimately, it was successful to separate the fungal luciferase by gel electrophoresis, selection of tris-glycine running buffer with 2% agarose matrix was the best (Fig. 11). Nevertheless, the supply of luciferin used for developing native agarose gels is an issue in these experiments. Initially, equisetumprone extracts were used to generate light during luciferase preparation and gel imaging. Unfortunately, beginning in October of 2015, extracts from the horsetail strobili stopped yielding functional equisetumprone. At this point, in order to observe light, 3-hydroxyhispidin had to be used for the developing solution, which was in short supply at that time. To decrease the assay size, a vertical gel system was used to overcome sample size with 1 mm thick gels. Proteins were extracted from agarose gel pieces by a series of warm buffered dilutions. The resulting sample was methanol:chloroform precipitated and digested with trypsin. These samples were analyzed by mass spectrometry and revealed no protein. A second batch of samples for mass spectrometry was similarly prepared, but equisetumprone was being reused once again. The second batch of samples were collected and sent to the private company (Veritas in Ribeirão Preto) for trypsin digestion and collection of spectrum data. Results reported in bioinformatics section.

Figure 11. In gel luciferase activity following electrophoresis in 2% agarose gel (tris, glycine). Gel segments had to be cut from gels for development with hispidin or equisetumpyrone. Upper left, cut and trimmed thicker horizontal gel segments. Upper right, outline of developing tray (green) and cut gel segments (red) of 30s dark photography with GE Imagequant with maximum light detection settings, vertical gel segments were developed with 3-hydroxyhispidin. Lower left, vertical gel segments developed with equisetumpyrone. Lower right, uncut vertical gel with loading wells removed developed with equisetumpyrone.

In addition to the second batch of agarose gel electrophoresis samples, ion exchange chromatography was used to further purify luminescent fractions. Fractions were collected from five runs of the ion exchange column with a 0-100% 1M NaCl gradient. A volume of 50 μ L of each 250 μ L fraction was combined with 15 μ L of equisetumpyrone and light was detected for 1 min. The brightest fractions were frozen after collection. We submitted 3 pooled samples of ion exchange chromatography fractions for trypsin digestion and protein mass spectrometry.

Submitted samples were selected for having high luminosity from each run and then pooled based on relative retention time. Samples (Table 10) were sent to Veritas for protein mass spectrometry sequencing, results presented in bioinformatics section.

Table 10: DEAE ion exchange chromatography samples for protein mass spectrometry. Fractions from eight FPLC runs were merged based on elution pattern and luminescence intensity. Sample numbers indicate the FPLC run and fraction number.

Pooled Sample ID	Sample	Concentration	1min RLU sum
IEC 1	1 – 12	0.366	26,185,380
IEC 1	3 – 18	0.102	1,735,520
IEC 1	4 – 18	0.411	5,112,500
IEC 2	5 - 35	1.842	5,191,935
IEC 2	6 - 36	0.6345	1,783,240
IEC 3	4 – 13	0.081	999,965
IEC 3	5 - 27	1.455	3,362,905
IEC 3	6 - 29	0.8325	1,386,320

4.2.4. Equisetumpyrone and fungal β -glucosidase

As mentioned in the previous sections, equisetumpyrone proved to be an excellent source of luminescent active molecules. We confirmed the presence of equisetumpyrone by mass spectrometry. Moreover, we were able to prepare a HPLC purification enrichment of equisetumpyrone. During this time period, an additional step was tested and subsequently added to luciferase enzyme preparation, calcium chloride precipitation of the luciferase from crude extract with the aid of mid-speed centrifugation (30,000 x g for 30 min). Equisetumpyrone addition to the solubilized CaCl_2 pellet resulted in minimal light emission compared to equisetumpyrone addition to ultracentrifuged pellet, with no CaCl_2 or crude protein extract. Nonetheless, addition of 3-hydroxyhispidin to the CaCl_2 pellet results in abundant light emission. The equisetumpyrone:luciferase (CaCl_2) solution resumes light emission by the addition of the CaCl_2 supernatant solution (Fig. 12). Purified recombinant β -glucosidase (courtesy of the

laboratory of Prof. Sandro Roberto Marana, IQ-USP) was also capable of restoring light emission to the equisetumprone:luciferase (CaCl_2) solution.

Figure 12. Reaction of equisetumprone with CaCl_2 prepared luciferase (blue) followed by the addition of the β -glucosidase containing CaCl_2 supernatant (red).

A diluted solution of equisetumprone was sprayed directly onto the surface of a mycelium culture of *N. gardneri* and light photographically observed - light emission of *N. gardneri* was strongly inhibited. Cultures were grown on agar media with a porous cellophane dialysis membrane separating the mycelium from direct contact with the agar. This way the mycelium was transferred from agar to the surface of a 2 mL bead of water, to which 100 μL of a equisetumprone solution was added. Mycelium dialysis membrane sheets were photographed over 2 h, while floating on dilutions of equisetumprone and equisetumprone-incubated with a β -glucosidase (Fig. 13). The 10-fold dilution of equisetumprone, with and without β -glucosidase incubation, resulted in strong inhibition of light. Light emission of the live cultures responded similarly to the 100-fold dilutions, with and without β -glucosidase incubation, as to water. When

the 1000-fold equisetumpryone solution that had been incubated with β -glucosidase was exposed to the live culture, light emission started increasing after 45 min of incubation, compared to water.

Figure 13. Equisetumpryone addition to the mycelium of *N. gardneri*. EP1 was 10-fold dilution of the equisetumpryone stock, EP10 was 100-fold, EP100, 1000-fold. Similarly, equisetumpryone incubated with β -glucosidase were also tested G1, G10, G100. Outlined green regions (right) pixel counted autonomously of time lapse image capture of mycelium sheets with equisetumpryone and β -glucosidase-incubated solutions. Mycelium sheets were monitored for 2 h.

4.2.5. DNA extraction

Initially two DNA extraction methods were tested but yielded low quantity and/or low quality DNA: Promega genomic DNA kits, Invitrogen genomic DNA kits. These kits were based on the basic strategy of cell lysis and DNA solubilization and RNase treatment, proteinase-K digestion of proteins and cellular debris precipitation, followed by isopropanol/ethanol precipitation of DNA. The DNA yield following the basic kit protocols was low, yielding less than

a microgram per extraction. Additionally, the final ethanol precipitated DNA pellet was highly pigmented. Ethanol washes of the pellet helped to reduce the pigmentation problem, but further decreased the DNA yield. Phenol/chloroform washes following protein precipitation worsened the problem, specifically with lower DNA yields or complete loss of DNA. High molecular weight polyvinylpyrrolidone (PVP), which had been reported (Balijja et al. 2008) to remove melanins during DNA extraction of deeply pigmented fungal tissue, also did not work with our cultures. At this point, the Promega and Invitrogen kits were abandoned for handmade solutions with focus on the lysis solution. Lysis buffer with 2% SDS initially showed promising results as DNA yield improved to over 5 µg per extraction, but the extracts were still highly pigmented. Phenol/chloroform washes resulted in complete loss of DNA. In addition to SDS other detergents were tested as well, including Triton X-100, CTAB and Sarkosyl, however SDS alone showed the best results.

Qiagen DNeasy kits, which involve binding DNA to a silica column, washing, and then elution of DNA from the column, were partially successful, yielding unpigmented and pure DNA. However, the final quantity of DNA was very low and would have required at least 20 kit extractions to collect only 10 µg of DNA required for genome sequencing of each sample. At this point, a DNA extraction protocol for Chestnut leaves, developed by the Clemson University Genomics Institute staff was attempted. This protocol involved an initial mild cell lysis with CTAB to release nuclei and mild centrifugation to pellet the nuclei. The nuclei were then washed in a complex solution including Sarkosyl, prior to additional CTAB being added with NaCl. The unmodified Chestnut protocol had higher yields than previous promising protocols, but still had lower than desired yields and DNA pellets were still pigmented. During an experiment with varying Sarkosyl concentrations a upper phase appeared in the solution, during the 65°C proteinase-K incubation. The upper phase was highly pigmented and the lower phase more

clear. The two phases were sharply separated, the upper pigmented phase was very viscous and became thicker when cooled to room temperature, while the clearer bottom phase was more fluid. Ethanol was added to both separated phases and centrifuged, no precipitation occurred in the pigmented phase, however in the clear aqueous phase an off-white pellet was formed. Additional ethanol washes and precipitations did not substantially decrease the pellet size. Solubilization of the pellet in water and subsequent nanodrop measurement, restriction enzyme digestion and gel electrophoresis, revealed that DNA was in high yield, digestible and therefore enzymatically labile, and free of RNA. While pigmentation had been greatly reduced there was still mild-pigmentation, attempts to remove this by phenol/chloroform resulted in complete loss of DNA.

The positive results from the Chestnut extraction protocol, specifically the formation of the biphasic solution with contaminating pigments pulled into the upper viscous phase leaving the bottom aqueous relatively pigment free and DNA rich, was optimized by removing solution components and steps that were ultimately unnecessary for phase separation. Finally, it was determined that a 6:5 ratio of CTAB to Sarkosyl and 1M NaCl helps the sharp phase separation and increase the volume of the aqueous phase. This extraction protocol was very successful for the species required for this genomics study: *N. gardneri*, *Lentinula edodes*, and *P. stipticus*, but it was not successful to remove all pigments from *O. olearius* (Fig. 14, 15). Phases formed upon NaCl and CTAB addition to the *O. olearius* extract but both phases were equally pigmented. Additional CTAB/Sarkosyl washes of the *Omphalotus* extract did not greatly decrease pigmentation and the resulting DNA yield was low and very impure.

Figure 14. Phase separation of CTAB:NaCl:Sarkosyl DNA extraction solution of six different fungal samples, from left to right: *N. gardneri*, *O. olearius*, *L. edodes*, *P. stipticus*-Blount, *P. stipticus*-Turkey, *P. stipticus*-Polk. The lower phase of all samples except *O. olearius* are ready for subsequent ethanol precipitation and residual Sarkosyl removal. The *O. olearius* sample required an additional two CTAB:NaCl:Sarkosyl separations to remove most dark pigments but DNA yield was still low.

	Conc. (ng/μl)	A260/A280	A260/A230
BL	330	1.88	1.47
TU	256.7	1.94	2.06
NG	215.8	1.83	1.56
LE	569.3	1.93	1.72

Figure 15. Top, digested and undigested genomic DNA samples on 1% agarose gel, 1 μg DNA in each lane. Bottom, qubit sample concentrations and nanodrop ratios of genomic DNA samples.

4.2.6. RNA extraction

Mycelium samples for RNA studies were grown the same way as the mycelium for DNA studies with the addition that frozen samples were collected at 8:00PM. RNA was simply extracted from 100 mg of frozen and ground mycelium with Qiagen Plant RNeasy Kits following the standard protocol. RNA samples were quantified and qualified with nanodrop and BioAnalyzer (RIN >8.0, Table 11).

Table 11: RNA samples for Illumina HiSeq sequencing grouping samples by family, species, strain, and replicate number. Concentration was measured by nanodrop spectrophotometry and RIN determined by Bioanalyzer.

Family	Strain	sample ID	Conc. ng/μl	260/280	260/230	RIN
Marasmiaceae	<i>L. edodes</i>	LE 3	710.3	2.09	1.9	8.8
		LE 6	278.5	2.14	1.95	8.2
		LE 7	309.9	2.14	2.39	9.1
	<i>N. gardneri</i>	NG 1	518	2.1	2.27	9.9
		NG 2	574.6	2.09	1.6	9.8
		NG 4	655	2.07	1.78	9.9
Mycenaceae	<i>P. stipticus</i> , Blount County TN, USA	BL 1	305.8	2.12	1.96	9.1
		BL 7	276.5	2.11	2.07	9.5
		BL 8	582.8	2.09	2.38	10
	<i>P. stipticus</i> , Turkey	TU 9	393.4	2.13	1.78	9.9
		TU 13	452.8	2.12	2.03	9.6
		TU 14	820.6	2.18	2.22	9.8

4.2.7. Genomic and Transcriptome Sequencing and Raw data analysis

The production of light from natural bioluminescent mushrooms occurs exclusively in the Agaricales order from at least three different families and use 3-hydroxyhispidin as a luciferin to produce light, which we sought to identify by sequencing the genomes and transcriptomes from healthy agar cultures of luminescent *N. gardneri*, and *P. stipticus* Blount co, and non-luminescent *P. stipticus* Turkey and *L. edodes*. These species will be hereafter referred by

the following abbreviations: NG, LE, BL or TU. These abbreviations are also used to distinguish sequence information titles in the python database. Collectively, when referring to all four, BL, TU, NG and LE, they are abbreviated as BTNL. NG and LE are both from the Marasmiaceae family with LE being a non-luminescent control for NG. BL and TU are the same species of *P. stipticus*, BL being the luminescent variety. We were expecting, based on comparable v1 genomes available on JGI servers (Table 12) to have 40-60 Mbp sized genomes with less than 2,000 scaffold sequences and a N50 around 150 kbp. Genome sizes were nominal, our scaffold assembly sequence count was exceptionally lower with corresponding greater average scaffold sequence length (Table 13) suggesting better than average genome assemblies for Agaricales species.

Table 12: Basic genome statistics of published genome projects of relevant related species.

Genus	Genome Size (Mbp)	No. of scaffolds	N50 (kbp)	No. of Genes	ref.
<i>Panellus</i> (Korea)	53.17	1562	181	15860	JGI
<i>Gymnopus</i>	89.1	2516	144	29375	JGI
<i>Moniliophthora</i>	75	7065	-	2002	Mondego 2008
<i>Omphalotus</i>	28.15	868	199	8172	Wawrzyn 2012
<i>Galerina</i>	59.42	414	-	28461	Riley 2014
<i>Armillaria</i>	58.35	4377	36.7	14473	Collins 2013

Transcriptome sequencing was eventless producing usable raw sequence read data. The assembled transcriptome sequences were on average long with high N50 values (Table 14). The number of assembled transcripts did vary between samples and assemblers. *L. edodes* had less identified transcripts compared to other species, whereas BL, TU, NG had quite similar unique transcript variety. Due to the complications in developing an effective DNA

extraction technique, transcriptome HiSeq sequencing finished before the genome MiSeq sequencing, and thus the initial batch of analyses were based on only transcriptome data as starting information. Much of the pathway and gene discovery efforts prior to genome completion was restarted once genome data was available.

Table 13: Assembly Genome results from Paired-end and mate-pair sequencing of luminescent and non luminescent *P. stipticus*, *N. gardneri*, and *L. edodes*.

	BL	TU	NG	LE
Luminescence	+	-	+	-
Organism	<i>P. stipticus</i>	<i>P. stipticus</i>	<i>N. gardneri</i>	<i>L. edodes</i>
Location	Blount Co.	Turkey	Piauí	Com. edible
Gapped genome size	39.0 Mb	41.7 Mb	46.6 Mb	49.6 Mb
No. of Scaffolds (_scaffold_)	571	869	293	1751
Scaffold N50	395 kb	318 kb	540 kb	71 kb

Table 14: HiSeq Transcriptome sequencing and assembly results of luminescent and non luminescent *P. stipticus*, *N. gardneri*, and *L. edodes*.

	BL	TU	NG	LE
Raw reads	65191457	57355442	69911396	69256224
No. of Bowtie seqs	23966	23446	24104	19593
No. of Trinity seqs. (_tr_)	41823	65808	52552	36787
N50	2712	2714	3039	2991

The four transcriptomes were screened against the currently available Agaricales genomes on NCBI and JGI servers by megablast (Evaluate < 0.005, wordsize 11) to identify genomes with high levels of sequence identity with the individual transcriptomes and among all four of the transcriptomes (Table 15). The two *Panellus* transcriptomes, unsurprisingly, matched most closely with the recently sequenced *P. stipticus* genome of a Korean isolate, which is likely

non-luminescent (Grigoriev et al. 2012, Jin et al. 2001). The *N. gardneri* transcriptome matched most closely with the *O. olearius* genome. The *L. edodes* transcriptome matched most closely with the *G. androsaceus* genome, in fact *G. androsaceus* is either the first or second closest genome match for all four transcriptomes. The genome of the luminescent fungi *A. mellea* also matches fairly well with all four transcriptomes. These genomes and the other genomes from NCBI and JGI servers are well annotated with predicted gene models and coding domain sequence regions, and often thorough functional annotations (GO, IPS, and KEGG).

Table 15 Megablast (wordsize = 11, evalule < 0.005) comparison of transcriptome sets against available Agaricales genomes on NCBI and JGI servers (35 total, top 16 displayed).

<i>L. edodes</i>			<i>N. gardneri</i>			<i>P. stipticus (BL)</i>			<i>P. stipticus (TU)</i>		
Species	Transcripts with blastn hits	Total blastn hits	Species	Transcripts with blastn hits	Total blastn hits	Species	Transcripts with blastn hits	Total blastn hits	Species	Transcripts with blastn hits	Total blastn hits
Gy_androsaceus_JB14	14044	33598	Olearius	16861	21375	Pa_stipticus_KUC8834	22596	45648	Pa_stipticus_KUC8834	22705	43225
Gymnopus	13780	23605	Gy_androsaceus_JB14	14633	29301	Gy_androsaceus_JB14	9101	17683	Gy_androsaceus_JB14	9605	18128
Olearius	10161	12334	Gymnopus	14256	21279	Galerina	9092	12109	Galerina	9586	12630
Mo_roreri	9253	13347	Mo_roreri	12671	18515	Ar_mellea	9091	15194	Hypholoma	9516	13273
Ma_fiardi	8813	12499	Ma_fiardi	11908	16355	Hypholoma	9022	12620	Ar_mellea	9502	15174
Ar_gallica	8530	11894	Ar_mellea	11630	18636	Ar_gallica	9007	13449	Ar_gallica	9489	13506
Ar_mellea	8489	13708	Ar_gallica	11566	15930	PI_crispa	8984	11678	Gymnopus	9439	13341
Gu_necrorhiza_MCA	8219	10667	Gu_necrorhiza_MCA	11200	14533	Plicaturopsis	8984	11678	PI_crispa	9390	11948
Galerina	7894	10406	Pa_stipticus_KUC8834	10803	15223	Gy_chrysopellus	8857	12044	Plicaturopsis	9390	11948
Pa_stipticus_KUC8834	7870	10799	Galerina	10755	14002	Gymnopus	8849	12715	Gy_chrysopellus	9335	12741
Gy_chrysopellus	7744	9998	Gy_chrysopellus	10527	14128	Mo_roreri	8679	12171	Olearius	9209	11344
Flammulina	7593	9671	Pleurotus	10267	12669	Pleurotus	8630	11069	Gu_necrorhiza_MCA	9137	11996
Pleurotus	7570	9136	Hypholoma	10263	13616	Gu_necrorhiza_MCA	8629	11521	Mo_roreri	9117	12627
Tr_matsutake	7567	14720	PI_ostreatus_PC9	10225	12828	Olearius	8576	10553	Pleurotus	9020	11248
Hypholoma	7549	10079	Tr_matsutake	10184	15895	PI_ostreatus_PC9	8566	11170	PI_ostreatus_PC9	8988	11474
PI_ostreatus_PC9	7548	9344	Flammulina	10174	13140	Tr_matsutake	8539	17891	Tr_matsutake	8888	19166

4.2.8. In silico dissection of hispidin metabolism

The genome assembly scaffolds (Table 16) were passed through the MAKER software, which predicted genes based on a number of other algorithms - mainly Augustus. Around 1,400 genes were identified in each genome (Table 16). CDS regions were passed through Blast2Go, InterProScan, and various EMBOSS algorithms for further functional and structural annotations. Transcriptomes were mapped to the genome with megablast, with 94% of mapped

transcriptome transcripts matching to MAKER predicted genes. A Python interface was written for the data allowing for browsing of the genome/transcriptome assembly and annotation data, the data structure was archived as super_scaffs python dictionary. In addition to the newly sequenced transcriptomes, a previously prepared transcriptome from *N. gardneri* (referred to as PI transcriptome) was mapped to the NG genome. The PI transcriptome set, which analyzed changes in transcript abundance between surface and submerged mycelium mats as a ratio of relative expression.

Table 16: Predicted gene model annotations of newly sequenced genomes of luminescent and non luminescent *P. stipticus*, *N. gardneri*, and *L. edodes*. Genes were predicted with MAKER and annotations assigned to genes with InterProScan and KEGG.

	BL	TU	NG	LE
MAKER pred. genes (_mg_)	13776	14995	15450	13428
Avg. gene length	1849bp	1768bp	1836bp	1786bp
Avg. exons per gene	6.2	5.9	6.3	5.9
Avg. transcripts per gene	1.5	1.4	1.3	1.1
GO annotated genes	6940	7200	6850	7429
InterPro annotated genes	8353	8637	8350	9358
pFam annotated genes	7409	7586	7447	8232
KEGG annotated genes	2668	2627	3087	3046

4.2.8.1. Phenylalanine ammonia lyase

The InterPro record IPR005922 is for phenylalanine Ammonia-lyase (PAL). This record was found in all genomes. Based on the literature, there should only be two PAL genes per genome, the InterPro record annotations were not consistent with BL and TU both having multiple genes with the PAL annotation and LE only having one. Therefore, a homology based search was initiated, using the CDS sequences of known PAL genes from other Agaricomycetes

species *A. bisporus*, *M. roreri*, *C. cinerea*, *L. bicolor*, *S. commune*, *S. lacrymans*, and *C. puteana* with the last two species being boletes. A multiple sequence alignment was generated of the seed PAL sequences, the two PAL genes from each species separated into two groups among the Agaricales. A consensus sequence was made from each group of PAL gene seeds and used to blast the BTNL transcriptomes to reveal the genes in the genomes. Two genes were found from BL, TU, and NG, with only one PAL gene in LE. Adding these genes into the multiple sequence alignment of seed PAL genes reveals PAL1 and PAL2 (Fig. 16). It appears LE has lost its second PAL gene.

Figure 16. Evolutionary tree of phenylalanine ammonia lyase (PAL) genes in Agaricales, two clusters are observed (red boxes). PAL genes sequenced in this study are in orange boxes.

In the *Panellus* samples, PAL transcript abundance is greater in the luminescent variety than non-luminescent for both PAL genes (Fig. 17). For the marasmoids (NG and LE) this is not the case, there is less transcript abundance in these two samples compared to the *Panellus* samples, but also the transcript abundance from the single LE gene is about equal to the combined transcript abundance from the NG PAL genes. Given the low expression of PAL in NG and LE a search for tyrosine ammonia lyase and other aromatic amino acid lyases was performed, none of these genes that were identified had significantly greater expression than the identified PAL genes. Additionally the PAL transcripts from the PI dataset were found, in luminescent surface cultures PAL expression was higher with the PAL1 (NG_mg_006822) PI transcript 2-fold greater while the transcripts from the PAL2 (NG_mg_003499) gene were degraded in the PI set.

PAL

Figure 17: Total transcript abundance of PAL genes, unique transcript sequence count for each gene in parentheses.

4.2.8.2. C4H and other Cytochrome P450s

C4H

From literature and browsing the JGI fungal database, genes annotated as C4H (EC 1.14.13.11) were found in 4 mushroom forming species, *Agaricus bisporus*, *Pleurotus ostreatus*, *Schizophyllum commune* and *Phanerochaete chrysosporium*. *S. commune* had only

one gene while the others had 2 genes. The single gene of *S. commune* has high sequence similarity with *A. bisporus* and *P. ostreatus* pairs and one of the *P. chrysosporium* genes, the other *P. chrysosporium* C4H gene is onto a clade of its own. The basis of C4H annotation for the seed genes may be questionable. All of them are indeed P450 genes, but the assignment of C4H is based on some association with the Arabidopsis C4H gene. The nature of this association is not clear and experimental verification of this assignment in fungi has not been performed. In addition, de novo annotation of our genomes with the online KEGG annotator (Moriya et al. 2007) using the Arabidopsis database did not assign the C4H annotation to any of our genes. Moreover, P450 genes have an overall conservation of structure, but their sequence and therefore functional specifics are not necessarily conserved. It could be very likely that these C4H genes, identified in fungi based on similarity to Arabidopsis genes, do not catalyze the same reactions. There were 10-13 C4H seed matches from each of the BTNL genome gene sets. The BTNL genes can be grouped into two based on homology matching to the various seed sequences. Group 1 have high sequence identity with nearly all seed sequences. Evidence for the second group is weaker, but consistently match with the *P. chrysosporium* gene for C4Hb or *S. commune* gene. Group 1, C4H genes from luminescent strains have higher transcript abundance than non-luminescent and some C4H genes in non-luminescent strains have antisense transcripts along with degraded sense transcripts (Fig. 18).

Duplication seems common for Group 1 CH4 genes with several nearly identical C4H genes in each genome. In the *P. stipticus* genomes one of the duplication events resulted in two C4H genes in close proximity to each other on the same chromosome (Fig. 19) . This C4H duplication is also observed in the *A. bisporus* and *P. ostreatus* genomes. This is not observed in the LE and NG genomes, where each of the C4H genes are on separate chromosomes. This

suggests that this duplication occurred at some point after the divergence of the Agaricoid, Tricholomatoid, and Pluteoid clades from the Marasmoid clade.

Figure 18: Total transcript abundance of identified C4H genes, unique transcript sequence count for each gene in parentheses.

Attempts were made to find orthologous pairing of the different C4H genes among the BTNL genomes, as sequence similarity among the C4H genes was high and not able to clearly distinguish orthologous groups by direct sequence alignment, the local gene space of regions neighboring a given C4H gene in a scaffold were compared among BTNL genomes, C4H genes with similar neighboring genes on different genomes were considered homologous. Unsurprisingly, there is a high degree of gene space conservation for *P. stipticus* Group 1 C4H genes. All 5 genes have clear homologues between the two sets. The double C4H genes on BL_scaffold_57 and TU_scaffold_70 had higher levels of transcription compared to their isolated counterparts. But overall, C4H transcription was much greater in BL than TU (Fig. 18).

Figure 19. C4H gene colocalization in *P. stipticus*. Blue and cyan genes are C4H cytochrome P450 genes, top is the full length DNA scaffold with a subset below for both BL and TU. Other genes of interest are also highlighted such as PAL (yellow), 4CL (orange) and type I PKS (green).

Gene space comparison of NG and LE group 1 C4H genes was less clear. Firstly, NG had two isolated group 1 C4H genes while LE had 4. The 4 LE C4H genes had unique gene spaces on their scaffolds suggesting the doubling was not a result of the diploid nature of dikaryotic fungal genome. Second, only one homologous C4H relation that could be suggested between NG and LE found on NG_scaffold_1 and LE_scaffold_4 both have the same adjacent gene an isoprenoid synthase (IPR008949). The expression of group 1 C4H genes in NG and LE is very different (Fig. 18), both NG group 1 C4H genes have strong expression but the gene on NG_scaffold_1 has an order of magnitude greater expression than the other and the group 2 C4H genes. Conversely, all four LE group 1 C4H genes have almost no transcript abundance. LE group 2 C4H genes have much stronger expression.

Comparisons of group 1 C4H gene topographies between tricholomatoid and Marasmius clades did not encounter any obvious homologies among all four genomes, although there is weak evidence that the C4H genes on LE_scaffold_4, NG_scaffold_1, BL_scaffold_36, and TU_scaffold_29 are orthologs. There appears to be conservation of the 3 solitary group 1 C4H genes from *Panellus* with three of the LE genes.

C3H and H3H

Figure 20: CYPomes. The heat maps are Smith-Waterman local sequence alignments among all genes that BLASTp matched CYP seed genes from FCPD. Match CYPs were filtered and reassigned when possible due to MAKER gene prediction (smaller heat map), perfect match is yellow, red has no significant Smith-Water similarity. A. BL - Blount co. Tenn. *P. stipticus* CYPome, B. TU - Turkey *P. stipticus*, C. NG - *N. gardneri*, D. LE - *L. edodes* CYPome.

The reaction of the coumaroyl-3-hydroxylase and hispidin 3-hydroxylase are similar. The first enzyme hydroxylates the phenol moiety and the second an alpha-pyrone. Although they are likely separate enzymes, the search strategy for each is almost identical based on available annotations and likely similarity to other characterized P450 genes that catalyze similar reactions. Seed genes for EC 1.14.13.36 - coumaroylquinase (coumaroylshikimate) 3'-monooxygenase, EC 1.14.13.88 - flavonoid 3',5'-hydroxylase and EC 1.14.13.21 - flavonoid 3'-hydroxylase were obtained from fungal genome databases and BTNL homologues were searched. The majority of resulting homologous matches were previously identified as C4H genes. As mentioned before, the sequence similarity of P450 genes is high, and at least in plants there is sequence similarity between some C4H and C3H genes. Since many of the identified BTNL genes are both associated with C4H and C3H seeds, it is impossible to determine further functional characterization without experimental evidence. Given the general

importance of P450 genes and the involvement of P450s in at least two steps of fungal luciferin biosynthesis, identification and organization of the P450omes was attempted in the BTNL genomes. The known P450 gene sequences from Agaricales were downloaded from the Fungal Cytochrome P450 Database (Park et al. 2008) and were BLAST(x)ed against the translated BTNL genes. Identified BTNL P450 genes were then examined for sequence similarity among themselves with Smith-Waterman local alignment (Fig. 20), sequence length, and some annotations in order to exclude non-P450 genes. The reduced P450ome genes were then aligned and a phylogenetic tree was generated in order to group evolutionarily similar P450 genes (appendix A).

4.2.8.3. 4-coumaryl:CoA-ligase

Annotations specifically for 4-coumaryl:CoA-ligase include, EC:6.2.1.12, IPR0102743, and GO:0016207. When these terms were queried against the annotated BTNL only the Enzyme Commission number matched the three genes in the database, one from each species except *N. gardneri*. Moreover, numerous 4CL genes in the literature (Wawrzyn et al. 2012, Weijn et al. 2013) and JGI servers have been identified in other agaric species. The sequences from 7 genes from *O. olearius*, 14 genes from Korean *P. stipticus*, and 2 genes from *G. androsaceus* were obtained and together with the three identified BTNL genes were blasted (blastp) against the full BTNL database genes. Blast match hits were evaluated by several criteria: number of seeds that matched the hit, hit annotations and transcriptome mapping (Fig. 18). Four 4CL genes were found in shiitake and five 4CL genes were found in *N. gardneri*, overall there was higher 4CL expression in *N. garneri*. In *P. stipticus* transcript abundance of 4CL genes was higher in the luminescent variety than in the non-luminescent one - the non-luminescent variety had more identified 4CL genes.

4CL

Figure 18: Total transcript abundance of 4CL genes, unique transcript sequence count for each gene in parentheses.

4.2.8.4 Hispidin synthase: Polyketide synthases

Type III PKS

A word-based search of the interproscan term names found the term IPR011141 for “Polyketide synthase, type III”, which identified a single gene found in both *Panellus* genomes (BL_mg_008168, TU_mg_008854). These genes also had other IPS terms chalcone/stilbene synthase, N-terminal (IPR001099) and chalcone/stilbene synthase, C-terminal (IPR012328).

pBLAST of these proteins against the other maker predicted genes from the BTNL genomes and tBLASTn against the assembled BTNL transcriptomes did not identify any other matching genes in the NG and LE sets, even with very low BLAST thresholds. A search on the JGI genome database for IPR011141 in other agaricomycotina found the annotation seldom used with only 9 species having genes matching this annotation. Most species with type III PKS annotations were polypores, with only the Korean *P. stipticus* genome representing the Agaricales from the JGI database. Similarly, BLASTx of this gene against the NCBI nr database found numerous homologous genes but mainly from fungal species outside of the Agaricales order and not found in any fungal species known to be bioluminescent. The expression of the *P. stipticus* gene with the IPR011141 annotation is higher in TU, but it is low overall in both species (BL_mg_008168: 4.7 , TU_mg_008854: 18.3).

HMG-CoA synthase

Type III polyketide synthases are part of the KASIII-PKSIII subfamily of thiolases, also found in this family is hydroxymethylglutaryl-coenzyme A synthases (HMG-CoA synthase). Both type III polyketide synthases and HMG-CoA synthases share a common mechanism for the acetylation of a substrate with ATP and malonyl-CoA. There was one HGM-CoA synthase found in each genome using the IPS term IPR010122. The four genes for HMG-CoA have gene expression patterns expected for bioluminescent and non-bioluminescent samples with higher transcript abundance in the BL and NG sets versus the TU and LE sets respectively. Although the expression in *P.stipticus* is much lower than NG and LE, BL is greater than TU, which had none. NG's expression of the PI data set was: L 341.05, NL 30.49. These genes also match with the type III PKS of *Agaricus bisporus* (AGABI2DRAFT_193269) and *I. obliquus* (KR069057), which

were initially found by querying the NCBI database with the phrase “type III polyketide synthase”.

HMG

Figure 19: Total transcript abundance of HMG:CoA synthase genes, unique transcript sequence count for each gene in parentheses.

Type I PKS

Plants make use of type III polyketide synthases in the production of chalcones and styrylpyrones, there is a possibility that fungi make use of a type I polyketide synthase for this task. Several genes in each genome were identified that have the traits of type I polyketide synthases by having the following annotations and domains: acyl carrier protein-like, polyketide synthase ketoreductase domain, polyketide synthase acyltransferase domain, thioesterase domain, dehydratase domain, polyketide synthase β -ketoacyl synthase, malonyl-CoA ACP transacylase, and ACP-binding domains. In general, the expression of PKSIs were higher in luminescent samples. The luminescent *P. stipticus* PKSIs with the highest expression, BL_mg_004963, is found in the phenylalanine secondary metabolism gene cluster on BL_scaffold_57. The gene NG_mg_009809 had the highest PKSIs transcript abundance in the NG genome, which is 3-fold higher than the highest LE PKSIs gene. NG_mg_009809 is upregulated in surface mycelium compared to submerged mycelium (PI: L:17.91,NL:1.04).

PKSI

Figure 20: Total transcript abundance of PKSI genes, unique transcript sequence count for each gene in parentheses.

4.2.8.5. Signs of fungal luciferase

Once hispidin (or bisnorynangonin) is formed by the polyketide synthase, it is then hydroxylated at C-3 position by hispidin 3-hydroxylase, likely a P450 (described in previous sections) and then reacts with the fungal luciferase to produce light. The fungal luciferase was partially purified and luminescing protein samples were sequenced by protein mass spectrometry. There were seven samples, three from ion exchange chromatography and four from agarose gel electrophoresis. Unfortunately, the ion exchange chromatography sample with the highest luminescence signal was contaminated with most fragments matching to human albumin and

keratin and very few BTNL genes. Overall the resulting fragments identified 754 *N. gardneri* genes with only 4 genes common to 6 of the 7 samples. Gene ontology terms of the matching genes were sorted and examined (Fig. 21). There was great diversity of catalytic activities (Fig. 21B) with many genes having oxidoreductase and hydrolase activities. There were 8 genes identified as monooxygenase activity (GO:0004497), all of them cytochrome P450 genes, including CYP genes previously identified as having similarity to C4H. One gene was annotated as being a dioxygenase, NG_mg_013493, but other annotations indicate that it acts on nitrogen groups and the expression of this gene and homologues in the *P. stipticus* and shiitake samples do not indicate its expression correlated with luminescence. The most frequent binding affinities (Fig. 21C) of the MS identified genes are metal ion binding (GO:0043167), FAD binding (GO:0050660), and purine nucleoside binding (GO:0001883) proteins. The major identified GO terms related to metabolic processes (Fig. 21D) in the MS identified genes are in carboxylic acid metabolism (GO:0019752), carbohydrate metabolic process (GO:0005975), and protein metabolic process (GO:0019538).

Four NG proteins were found in 6 out of 7 mass spectrometry samples analyzed, but they are likely not the luciferase, based on annotations. These genes are a porin, nucleoside diphosphate kinase, and two are glucosidases. Only one of the four genes had greater expression in the luminescent samples than the non-luminescent one. The remaining identified proteins by MS of *N. gardneri* genes were screened based on presence in mass spectrometry samples, functional annotations, blast match similarity with *P. stipticus* and shiitake genes and subsequent expression analysis and scaffold examination of neighboring genes. Of the genes identified by protein MS an epoxide hydrolase, NG_mg_003294 (Fig. 22), is the best candidate for the fungal luciferase. NG_mg_003294 and its homologues have higher expression in *Neonothopanus* than in shiitake, homologues found in the *Panellus* genomes have similar

expression levels. The annotations of NG_mg_003294 are most interesting suggesting it reacts with stilbene like molecules, the structure of hispidin is quite similar to stilbenes. A cytochrome P450 gene is in close proximity to NG_mg_003294 on NG_scaffold_3, no other phenylalanine secondary metabolism genes were found close to NG_mg_003294. Peptide fragments from NG_mg_003294 were found in one sample prepared from ion exchange chromatography and two samples from agarose electrophoresis.

Additionally, superoxide dismutase (SOD) and catalase (CAT) were identified in the samples of luminescent fungi. It is likely that none is acting as the fungal luciferase, they are of note as both genes have been previously been hypothesized as either being the luciferase or governing the chemiluminescent reaction by other groups (Shimomura 2006, Vydryakova and Bissett 2016). SOD identified by protein MS, NG_mg_001836, has much greater expression in *N. gardneri* than *L. edodes*, but has about equal expression levels in *P. stipticus*. CAT identified as NG_mg_004940 had almost no expression in *L. edodes* and both forward and some reverse transcription in *N. gardneri* - transcript abundance was greater in non luminescent *P. stipticus* than luminescent.

Catalytic activity GO:0003824

Binding GO:0005488

Figure 21. Protein Mass spectrometry identified protein GO terms

Figure 22. Cis-stilbene-oxide hydrolase found in the *N. gardneri* genome, gene ID NG_mg_003294. Upper shows position of gene on scaffold 3, with the local position directly below, red outline gene is NG_mg_003294. Mid, exons of the NG_mg_003294 gene with aligned transcripts directly below. Lower left, transcript abundance of homologues of NG_mg_003294 in BL,TU,NG,LE gene sets. Lower right, Go and Interpro/pFam annotations.

Figure 23. Superoxide dismutase found in the *N. garneri* genome, gene ID NG_mg_001836. Upper shows position of gene on scaffold 19, with the local position below. Red outline gene is NG_mg_001836. Mid, exons of the NG_mg_001836 gene with aligned transcripts below. Lower left, transcript abundance of homologues of NG_mg_001836 in BL,TU,NG,LE gene sets. Lower right, Go and Interpro/pFam annotations.

115

5. Discussion

5.1. The molecular nature of fungal luminescence

Five years ago it was only possible to perform the hot-cold assay in vitro with crude extracts of luminescent fungi, today the field has progressed with developments in the characterization of the luciferin, the luciferin biosynthesis, and the identification of the luciferase. Now it is possible to perform the light emitting reaction with purified 3-hydroxyhispidin, several plant extracts containing bioluminescent active compounds, and with luciferin precursors. While several strategies of investigation have been employed in my doctoral studies of fungal bioluminescence, from ecology (discussed in the second half of this discussion) to genomics, the most insight has come from the bioinformatics study of luciferin metabolism and biosynthesis. Moreover, the discovery that equisetumpyrone extracted from plants elicits light emission with fungal protein extracts makes the fungal system interesting through the lense of plant molecular biology.

5.1.1. The *panellus* phenylalanine secondary metabolism gene cluster

We have identified several genes in the genomes of *Panellus stipticus* and *Neonothopanus gardneri* that are directly involved in phenylalanine secondary metabolism, the major pathway responsible for hispidin biosynthesis. In *P. stipticus*, these genes exist in a gene cluster on scaffold 57 of the luminescent geographic isolate from Blount county Tennessee and on scaffold 70 from the non-luminescent isolate from Turkey (Fig. 25). The enzymes phenylalanine ammonia lyase (PAL), *p*-coumaryl hydroxylase (C4H), *p*-coumaryl CoA ligase (4CL), and a polyketide synthase (PKSI/SPS) convert phenylalanine to hispidin or bisnoryangonin. On BL_scaffold_57 there is a duplication of C4H, 4CL and PAL genes, followed by a single PKSI gene, and the same gene pattern is found on TU_scaffold_70. The topographical organization of the cluster is maintained among the two *P. stipticus* strains gene expression from this gene cluster is different in some cases. Both sets of 4CL and C4H like

P450 genes are strongly expressed in BL and TU. On the other hand, PAL has two full length genes in the BL variety, but no detectable gene expression from these genes, in TU the PAL more distant from the PKS1 gene is truncated in length but both PAL genes in TU have substantial expression. BL PAL genes in the phenylalanine metabolism cluster appear to be silenced, but other PAL genes found in different genomic loci in BL have non-zero and abundant expressions. One of the different BL PAL genes (BL_mg_004080) exists on a small scaffold (BL_scaffold_336) with no other genes and has the second greatest expression. The greatest difference between the BL and TU clusters is the expression of the PKS1 gene. In BL it is highly upregulated and in TU downregulated. The downregulation of PKS1 in the European variety of *P. stipticus* could explain why luciferin was not detected in non-luminescent strains by previous experimenters. In addition to the known phenylalanine secondary metabolism genes, there are three other genes that are conserved in both clusters. A histone acetyltransferase domain containing a ribonuclease-H gene is found downstream of PKS1. Moreover, a lysine methyltransferase and an uncharacterized gene are found between the PAL:C4H:4CL duplicates. All three non-phenylalanine metabolism genes found conserved between both BL and TU clusters have substantial expression, the methyltransferase and ribonuclease-H might be involved in cellular signaling and luciferase expression if located in a trans-location. This cluster is found in both *P. stipticus* genomes and likely plays a significant role in *P. stipticus* bioluminescence. When the entire cluster was searched with blastn against the NG genome no equally sized matches are found. Blastp of translated *P. stipticus* cluster genes against the translated genes of NG was performed to identify NG_scaffolds with multiple gene matches. The synteny of the *P. stipticus* cluster was low against the NG genome with the various phenylalanine genes that matches isolated genes in the NG genome. NG scaffolds with multiple

matches in close proximity to the phenylalanine secondary metabolism genes were typically P450 genes.

Figure 25: *P. stipticus* phenylalanine secondary metabolism gene cluster, black lines between scaffolds indicate strong sequence similarity. Vertical green bars next to gene indicate transcript direction and abundance, light green low expression dark green higher expression.

5.1.2. Clustering of other secondary metabolism genes

Clustering of genes involved in secondary metabolism appears to be common in fungi (Keller et al. 2005, Hoffmeister and Keller 2006). Several gene clusters were identified in *O. olearius* involved in sesquiterpenoid biosynthesis (Wawrzyn et al. 2012). A cluster of genes was found in *A. bisporus* with many genes involved in melanin biosynthesis (Weijn et al. 2013). Interestingly, the phenylalanine secondary metabolism pathway is involved in melanin biosynthesis, but those genes were scattered outside of the melanin biosynthesis cluster genes identified by Weijn et al. (2013). Much of the characterized clusters in literature are on a species level with little comparison to other species, it is unknown to what extent metabolism gene clusters are stable. The presence of the phenylalanine secondary metabolism cluster in *P. stipticus* and the complete absence of a similar cluster in *N. gardneri*, and *L. edodes* suggests that there is significant turbulence in the fungal genomes.

5.1.3. Cytochrome P450 genes

As mentioned before, there are several cytochrome P450 proteins involved in the hispidin biosynthesis pathway. Cytochrome 450s are a class of heme proteins that are highly conserved among all kingdoms of life and are responsible for many monooxygenation and hydroxylation events in the metabolism of diverse organisms. There has been great effort to characterize all P450 genes in a given species and numerous categorization and classification systems exist (Nealson 1996). Many Agaricomycotina species have more than 100 different P450 genes (Park et al. 2008), but the functional characterization of their roles in specific metabolisms and the reactions they catalyze have not been examined in any great detail. We have begun this process by identification of the P450 genes in our genomes and have sorted them on the basis of sequence similarity into conserved groups. The P450 genes that are structurally similar to the previously characterized C4H are of likely great importance to the

bioluminescence system in fungi, functioning as the C4H gene but also the C3H and perhaps the hispidin 3-hydroxylase, whose NADPH dependent hydroxylation mechanism is common among P450 enzymes.

5.1.4. Genomes and Transcriptomes

In this study the expression of genes was inferred from the HiSeq sequencing of cDNA sampled in triplicate, whose transcript abundance was determined from the assembly sequences. The assembled sequences were mapped to the genomes by BLAT. The genomes had previously been annotated by MAKER annotation pipeline, identifying gene protein coding regions. It had been noticed during the search for phenylalanine secondary metabolism genes that many MAKER genes had been partially called in error with one MAKER gene actually containing several independent genes. This had been noticed on a case by case basis, when it was observed that multiple non-overlapping BLAT mapped transcript sequences were mapped to a single MAKER predicted gene - whose transcript expression and annotations suggest independent genes. This is major a limitation of this version of the draft genomes and many other early version draft genomes.

An early two-stage comparison was made with the transcriptome data alone. In the primary comparison the luminescent species were compared to their respective controls, Marasmiaceae: BL vs. TU and Mycenaceae: NG vs. LE. The primary comparisons were then compared among themselves in the secondary comparison BL vs. TU and NG vs. LE, in hope to identify common transcripts, whose expression was greater in the luminescent species and lower in non-luminescent species across two family branches of the fungal evolutionary tree (Fig. 26). In the primary comparisons the two transcriptome sets were matched by blastn reciprocally (evalue < 0.005, wordsize 11) and in this preliminary analysis the blast results were grouped into three groups:

- 1) sequence pairs that are reciprocally top hits,
- 2) sequences that have hits in the other transcriptome, but do not have top hit partners,
- 3) transcripts with no hits in the primary comparison.

Figure 26: Transcriptome reciprocal blast analysis. Primary comparisons were blastn based, transcripts with direct and reciprocal blastn relations are colored blue and green, transcripts with hits but not reciprocal top-hits are yellow, red transcripts have no hits in the primary comparison. The fold change (FC) was calculated for transcripts with reciprocal hits, FC > 2.0 were colored lime green, and FC < -2 were colored red. The secondary comparison between Marasmius and Mycena (Panellus) primary comparisons was tblastx based, reciprocal hits are the thick black lines. Those transcripts identified in the secondary comparison had their expression ratios examined, first they were grouped into having similar FC in both primary (grey), having greater expression in the marasmius (blue) or greater expression in the mycena (green). Those transcripts with similar FC in both families were then further assessed for FC pattern, transcripts with FC < |2| remained grey, FC < -2 were associated with being downregulated in the bioluminescent condition (red), while FC > 2 were associated with being upregulated in the bioluminescent condition (lime).

The Marasmiaceae had 7,086 transcripts that were direct top-hit matches between both transcriptome sets of which 624 were upregulated in the bioluminescent condition with a fold-change ≥ 2.0 and 2,400 were downregulated with a fold-change ≤ -2.0 . The Mycenaceae

had 12,074 transcripts that were direct matches in both transcriptome sets, 1,162 were upregulated in the bioluminescent condition and 1,824 were downregulated. The secondary comparison between the Marasmiaceae and Mycenaceae transcriptomes were matched with tblastx (Evalue < 0.005, wordsize 4). The secondary comparison was grouped in two, the first group was between the transcripts with top-hit matches identified in the primary comparison, 2,897 transcripts were direct matches in all four transcriptome sets. The second group in the secondary comparison was between NG transcripts with no LE hits against BL transcripts without a top-match partner and BL transcripts with no TU hits against NG transcripts without a top-match partner, 1379 additional transcripts matches between MA and MY were identified. The secondary comparison identified a total of 4276 transcripts common between MA and MY transcriptome sets with direct and reciprocal top-hit matches. Expression values of the reciprocal top-hit matches were assessed by the log2 ratio of the MA and MY fold changes, log2 ratios were grouped, ≥ 2 are transcripts with greater expression in the MA set, ≤ -2 are transcripts with greater expression in the MY set, log2 ratios between 2 and -2 are transcripts with similar expression in both MA and MY. There were 2,648 transcripts with similar expression in both MA and MY, *i.e.* same fold-change in both primary comparisons. These were also grouped, yielding 492 transcripts upregulated in the bioluminescent condition ($FC \geq 2$), 17 downregulated ($FC \leq -2$) in the bioluminescent condition, and 2,139 with similar expression in both luminescent and non-luminescent conditions. In total, only 4,276 transcripts were common among all four species, this is far short of the on average 14,000 genes found in each genome. The loss of matches was due to the strict metrics of reciprocal blast, where multiple homologous genes that are strongly structurally similar can be lost or obfuscate the homology assignment. It was hoped that with genome data this comparison would have been more clear, where gene positional location along a DNA scaffold would aide in matching homologs. Synteny among the

species DNA scaffolds was low for most genes, although the situation was better between two *P. stipticus* genomes. The global comparison among all genes between the four sets based on gene expression data was largely unsuccessful due to the non-static nature of fungal genomes.

5.1.5. Luciferase Substrates

The fungal luciferase is known to emit light with two different luciferins biosynthesized by plants and fungi, 3-hydroxyhispidin and 3-hydroxybisnoryangonin (Purtov et al. 2016). Moreover, we have shown that numerous plants can also serve as sources of bioluminescent active compounds, with the strong light emission of equisetumprone from horsetail strobili to weaker but still substantial emissions from other plants (*Pistia*, *Arabidopsis*, and others). The plant substrates are likely glycosylated compounds similar to equisetumprone, which is 3-hydroxyhispidin with an 3-O-linked glucose moiety. The alternative kinetics seen with *Pistia* may be due to an alternative sugar modifications or the polyketide may be 3-hydroxybisnoryangonin, whose kinetics are known to be delayed (Purtov et al. 2016). Other plant extracts, such as pine and concentrated *Arabidopsis* extracts result in no light emission and seem to inhibit the fungal luciferase.

In some plants hispidin, the precursor of fungal luciferin (3-hydroxyhispidin), is biosynthesized as a result of a type III PKS performing two condensation reactions of malonyl-CoA as opposed to the normal activity of plant type III PKS, whose condensation of three malonyl-CoA and ring closure yields naringenin chalcone. Naringenin is formed by chalcone isomerase and is the major branchpoint in flavonoid biosynthesis. The numerous tertiary metabolites of naringenin are largely produced by the hydroxylation and dehydrogenation of the naringenin molecular backbone (KEGG pathway map00941). It is tempting to speculate that plants could be made bioluminescent by modification of the enzymes involved in phenylalanine secondary metabolism and flavonoid biosynthesis pathways. Likely

the type III PKS in most plants will have to be modified in order to undergo only two condensation reactions of malonyl-CoA. Additionally, the activities of other relevant enzymes would likely have to be modified, such as flavonoid-3',5'-hydroxylase and coumarate-5'-hydroxylase, which may be the same enzyme as they both act upon C-5 position. The activity of enzymes that act on the second phenyl ring in flavonoids, which is absent in hispidin must also be monitored as they may act upon the heterocyclic ring of hispidin.

We have performed experiments in collaboration with the research group of Ilia Yampolsky, using luciferin analogues, whose catechol moiety had been replaced by other groups. Alteration of this moiety changed the light emission spectrum and the kinetics of the light emission reaction (publication submitted). Nonetheless, 3-hydroxyhispidin and 3-hydroxybisanthranol resulted in the brightest total amount of light among tested analogs.

Plant isoflavonoids and the less common neoflavonoids should be examined for their activity with fungal luciferase (Fig. 27). This class of compounds might not be functional with the fungal luciferase, but they do present a similar alpha-pyrone moiety similar to hispidin and other luciferin analogues. The substitution of the 15 carbons of the neo/iso-flavonoid would govern the spectral capabilities of the resulting oxyluciferin luminescing fluorophore as it goes from excited state to ground state. While I do not believe this chemistry has been examined in plants it could be future avenue of plant luminescing engineered metabolisms, that would be aided by understanding the evolutionary models of other luminescent system luciferases such as fungi.

Figure 27: Possible botanical phenylalanine based bioluminescent systems. Through the action of styrylpyrone synthase (SPS) following phenylalanine secondary metabolism, hispidin like compounds (3-hydroxyhispidin, *left*) are formed. Alternatively in most plants chalcone synthase (CHS) converts phenylalanine metabolites to naringenin-chalcone an intermediate to numerous products including isoflavonoid (*center*) and neoflavonoids (*right*). The α -pyrone varieties of these classes of compounds could be potential luciferins undergoing chemiluminescence through an endoperoxide luminescence similar to fungal luminescence (right).

5.1.6. The regulation of fungal bioluminescence

With the recently sequenced genomes of *N. gardneri*, luminescent *P. stipticus* and other fungal genomes available on public servers, the regulation of bioluminescence can be further explored. It appears that the type I polyketide synthase (PKSI) is a major factor ruling bioluminescence in *P. stipticus*. It would be interesting to monitor the activities of PAL, CYP(CH₄), 4CL, and PKSI in the luminescent and non-luminescent tissues of *Mycena luxaeterna* and *Gerronema viridilucens*. The phenylalanine secondary metabolism pathway has been shown to be enhanced by nitric oxide (Zhao et al. 2015), NO production by fungi can be stimulated by co-culture with other fungi, this could explain the increased light observed at the front of a contamination in a culture of *O. olearius* (Fig. 28). Additionally, circadian rhythm studies can be further conducted with *P. stipticus* and *N. gardneri*.

Figure 28. *O. olearius* culture colliding with contamination growth, at the collision front light emission is increased.

5.2. Ecology discussion

5.2.1 Ecological significance of fungal bioluminescence

The question, why do fungi glow is more difficult to answer than how do they glow, although neither can be currently answered in full. The first question implies that the organism

benefits itself by glowing, which may not be the situation from an ecological perspective. There are numerous examples at the ecological level for beneficial advantages of bioluminescence (Haddock 2010). Bioluminescence in fungi could be advantageous at the metabolic level and/or it could be advantageous at the ecological level (Lingle et al. 1989, Bermudes et al. 1992). It has been long hypothesized that the light is a means to attract spore dispersal agents (McAlpine 1900, Ewart 1906, Johnson 1919, Lloyd 1974, Sivinski 1981), although there are those that have doubted upon this role (Murrill 1915, Buller 1924). After observing high numbers of insects and other arthropods on glowing mushroom baited glue traps, Sivinski (1981) expanded the ecological hypothesis list to include:

- (1) luminescence may be a method of attracting spore and fungal propagule dispersers
- (2) attraction of carnivores of fungivores
- (3) attraction of fertilizers
- (4) repulsion of negatively phototropic fungivores
- (5) attraction of fungivores of other fungal competitors
- (6) and the light may be an aposematic warning signal of mushroom toxicity.

We have presented here evidence supporting two of Sivinski's hypotheses, the attraction of spore dispersers, and the attraction of secondary predators. We have not directly observed the propagation of viable fungal propagules, but we have observed many animal species consuming and interacting with bioluminescent mushrooms. Domestic pigs were responsible for the bulk of mycophagy in the Piauí test area - on a given night it was not uncommon for them to consume 40% of monitored mushrooms (~25 mushrooms). While domestic pigs are a relatively new addition to the mushroom ecosystem in Piauí and not observed in the Atlantic Rainforest, it should be acknowledged that nocturnal mycophagy of mammals occurs and should be

monitored for. It is noteworthy that deers are known fungal propagule dispersers (Cazares and Trappe 1994). Cockroaches were the most commonly observed fungivores as they were observed eating bioluminescent mushrooms in the Atlantic Rainforest and in Piauí.

Sivinski also proposed that the light may function to attract predators of fungivores. This is indeed occurring with spiders as they have been observed many times using the fungal light as a natural lure to capture prey. This is reminiscent of the burglar alarm effect observed between dinoflagellates and dinoflagellate primary and secondary predators (Abrahams and Townsend 1993, Fleisher and Case 1995). In controlled studies with dinoflagellates capable of producing flash luminescence, their population density increases as the population of primary predators decrease because of the attraction of secondary predators. This results in mutual benefit for both the populations of dinoflagellates and secondary predators. This could also be occurring with fungal bioluminescence with the cockroaches as primary predators and spiders as secondary predators. This appears to be occurring in the niche around fungal bioluminescence, but it is not clear what effect this has on the evolutionary fitness or population dynamics of the players involved. Despite some nocturnal animals may be avoiding the mushrooms, overall they seem to be attractive to animals, thus it does not seem that bioluminescence is an effective aposematic signal.

The attraction of bioluminescent click-beetles to *N. gardneri* is interesting. Click-beetles are known to emit light in the green region similar to the light of bioluminescent fungi, and are also quite capable of detecting fungal luminescence (Seliger et al. 1982, Lall et al. 2010). The click-beetles would often arrive in pairs and crawl over the mushroom cap and gills. The activity of the beetles on the mushrooms could be a means of dislodging spores from the mushroom and dislodged spores could be adhered to the click-beetle surface. Whether this is actually

occurring remains unknown as this is the first report of luminescent beetles interacting with bioluminescent fungi.

Earwigs observed in bioluminescent fungi may be parasites as they were usually found living inside mushrooms. On the other hand, click-beetles and crickets seemed to spend some time at the mushroom and move along. Earwigs would fight off other arthropods that encroached the mushroom, including cockroaches, spiders and beetles. Perhaps the earwigs have a symbiotic defensive relationship.

Ants were found in all experimental conditions in great numbers, and were the most frequently observed arthropod in the infrared video. In general, they did not seem to pay much attention to the bioluminescent mushrooms, but were observed in statistically greater numbers around bioluminescent mushrooms in both the Atlantic Rainforest and Piau. While ants are largely known to navigate using scent smells, they also are known to use visual cues. The nocturnal ant *Myrmecia pyriformis*, which performs most navigation in twilight periods is known to have decreased navigation efficiency in the dark. The march of ants slower, less linear and they seem lost at night. These kinds of behavior is likely due to decreased signal-to-noise ratio of visual cues as upon sunrise they often find their way back to the nest (Narendra et al. 2012). Our observation of ants wandering around bioluminescent mushrooms may be a result of lost ants circling the visually distinct mushroom as the fungal light may be the only visual cue in the nocturnal forest.

5.2.2. Nocturnal observations of insects and bioluminescent mushrooms

The IR video is excellent for detecting and observing the behavior of animals greater than 1 cm. The detection of animals smaller than this is possible, but their identification is not. The combination of the infrared lighting and difficulty of focusing GoPro cameras on such small objects contribute greatly to this size limitation. While this size limitation resulted in some insects

not being detectable in Piauí, the problem is much larger for the data from the Atlantic Rainforest (Fig. 29). The combination of IR and glue trap results gives an overlapping and more complete understanding of the ecological niche surrounding bioluminescent fungi.

small ground

Figure 29: Merged timelines of small ground arthropods that could be detected in the IR video but not identified, timelines include data from the 2014 and 2015 Piauí expeditions and the 2014 Atlantic Rainforest expeditions.

In addition to the detection and identification limits of infrared timelapse, selection of appropriate control observations was non-trivial. In the Atlantic Rainforest numerous non-luminescent small mushrooms can be found in the full light of the day, but they are quite impossible to find at night with a flashlight - it is actually easier to spot luminescent mushrooms in darkness when present than similar non-luminescent mushrooms in the day time. In Piauí the situation was different, when *Neonothopanus* was present it was the major gilled mushroom found in the Coconut Forest, nearly all other mushrooms were polypores that are visually different and have a different texture. Juvenile babaçu palms without *N. gardneri* mushrooms were used as controls in most of timelapses performed in Piauí.

It is very likely that the nocturnal spread of spores and other bioluminescence propagules is enhanced by arthropods and other animals attracted to the light emission. Enhanced spore dispersal seems beneficial, but it may not make much difference as the small size of mushroom spores allows them to be very efficiently aerosolized and subsequently dispersed by the smallest of micro-currents - provided the forest counts on some airflow. Animal vectors may play an important role in some fungal life cycles, but the air is likely the main fungal

spore dispersal vector. The distance that spores travel by air dispersal follows a Power Law Model. The majority of spores only travel short distances, however less but still significant amounts of spores can travel longer distances away from the source via air current and brownian motion (Galante et al. 2011). The distribution depends highly on the total amount of spores released (Malloch and Blackwell 1992). It should be noted that bioluminescent fungi grow in many different locations and environments and the ecological significance of luminescence may be different in different species. The two species monitored in this study, *Mycena luxaeterna* and *Neonothopanus gardneri* are both found close to the surface of the ground and may rely more heavily on ground dispersers than other fungi. On the other hand, *Panellus stipticus* and *Mycena globulispora* are both found off of the ground on dead branches and twigs, often above head level. These species may rely more on the wind for spore dispersal. In summary, although air can disperse fungal spores, arthropods seem to play an important role as dispersers in the case of bioluminescent fungi - mostly in very dense forests where the airflow at ground level is limited. Arthropods represent an additional mechanism for spore dispersal and might grant to bioluminescent fungi some evolutive advantage.

In these studies we examined the above ground interactions of bioluminescent fungi with the environment, another avenue for bioluminescent fungal interaction with the environment remains totally uncharacterized, subsurface mycelium. Many bioluminescent fungi only emit light from subsurface mycelium in nature, such as the honey mushrooms (*A. mellea*). There are historical reports of excavated mycorrhizal mass that are known to glow. Hence, bioluminescent mycelium is likely common below ground. It has been reported that fungi that produce subsurface fruiting bodies are assisted in spore dispersal by burrowing animals (Cazares and Trappe 1994, Colgan and Claridge 2002, Johnson 1996). The green light of subsurface mycorrhiza may help retain or repel light sensitive sub terrestrial animals in the area, or the light

may be involved in some uncharacterized interaction of the fungal mass with the plant root mass in mycorrhize, whose effects may be apparent in the shoots of plants through root-shoot signaling.

6.0 Conclusions

Will science ever be able to definitively answer the ancient question of why do some fungi glow? Probably not. Fungi are horrible parents, producing millions of spores a day, normally relying on air-currents for spore dispersal and only needing a few spores to successfully germinate for the life cycle to be complete. Using light as means to attract propagule dispersers, as plants use color and flavor to attract dispersers of pollen and seeds, both of which are often heavier than fungal spores, is an attractive hypothesis but is difficult to test in order to understand the evolutionary significance of this trait. Presented here is evidence that numerous animals are attracted to the fruiting bodies of bioluminescent fungi at night: cockroaches, spiders, beetles, ants, earwigs, crickets and pigs, with each having a different interaction with the glowing mushroom. Future studies should investigate the fungal propagule carrying capacity of these potential spore dispersers. Additionally proposed in this work is the potential of a relationship of subterrestrial luminescent mycelium and plant roots in the form of mycorrhizae, with the green light of fungi having some form of effect on the plant. The effect of green light on plants is poorly understood, especially of roots and of mature trees. Luminescing fungi grow exclusively on plant material of various levels of decomposition. If green light on plant roots has some effect on growth, then fungi that glow could ultimately benefit from this. If green light is beneficial to plant health, then ultimately more plant material would be available to the fungus once the plant eventually dies. Alternatively, green light could be harmful to the plant, causing a more short-term increase in dead plant material for the fungi to consume.

On the other hand, the question of how fungi glow is almost understood at a chemical

and genetic level. The not uncommon fungal and plant metabolite hispidin is the precursor to the fungal luciferin, 3-hydroxyhispidin. While the work presented here did not ultimately identify the light generating and 3-hydroxyhispidin consuming fungal luciferase, a better understanding of the hispidin metabolic pathway was achieved. The metabolic production of hispidin in plants and fungi is strikingly similar with homologous genes for the conversion of phenylalanine to caffeoyl-CoA or coumaryl-CoA. Plants and fungi differ in the evolutionary origin of their styrylpyrone synthases: fungi use a Type I polyketide synthase and plants use a type III polyketide synthase. Additionally, at least in some bioluminescent fungi the genes involved in hispidin biosynthesis are clustered together in the genome. While the expression differences of the fungal luciferase remain unknown between luminescent and non-luminescent strains of *P. stipticus*, the lack of luminescence in non-luminescent *P. stipticus* is at least partially the result of the lack of expression of the type I polyketide synthase found in the common gene cluster which would ultimately result in a lack of hispidin. The future of fungal bioluminescence research and application looks quite bright.

7.0. References

Abe I, Morita H. (2010) Structure and function of the chalcone synthase superfamily of plant type III polyketide synthases. Nat. Prod. Rep. 27, 809-838.

Abrahams MV, Townsend LD. (1993) Bioluminescence in Dinoflagellates: a test of the burglar alarm hypothesis. Ecology. 74(1): 258-260.

Airth RL, Foerster GE. (1962) The Isolation of Catalytic Components Required for Cell-free Fungal Bioluminescence. Arch Biochem Bioph. 97, 567-573.

Airth RL, Foerster GE. (1964) Enzymes Associated with Bioluminescence in *Panus Stypticus* Luminescens and *Panus Stypticus* Non-Luminescens. J. of Bacteriology. 88(6): 1372-1279.

Airth RL, McElroy WD. (1959) Light emission from extracts of luminous fungi. J Bacteriol. 77, 249-250.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. (1990) Basic Local Alignment Search Tool. J. Mol. Biol. 215: 403-410.

- Awadh Ali NA, Jansen R, Pilgrim Horst, Liberra K, Lindequist U. (1996) Hispolon, a yellow pigment from *Inonotus hispidus*. *Phytochemistry*. 41(3): 927-929.
- Awadh Ali NA, Mothana RA, Lesnau A, Pilgrim H, Lindequist UA. (2003) Activity of *Inonotus hispidus*. *Fitoterapia*. 74: 483–485.
- Bae YM, and Hastings JW. (1994). Cloning, sequencing and expression of dinoflagellate luciferase DNA from a marine alga. *Gonyaulax polyedra*. *Biochim. Biophys. Acta* 1219: 449-456.
- Balaji NV, Ramani MV, Viana AG, Sanglard LP, White J, Mulabagal V, Lee C, Gana TJ, Egiebor NO, Subbaraju GV, Tiwari AK. (2015) Design, synthesis and in vitro cell-based evaluation of the anti-cancer activities of hispolon analogs. *Bioorganic and Medicinal Chemistry*. 23:2148-2158.
- Beckert C, Horn C, Schnitzler JP, Lehning A, Heller W, Veit M. (1996) Styrylpyrone biosynthesis in *Equisetum arvense*. *Phytochemistry*. 44(2): 275-283.
- Berkel WJH, Kamerbeek NM, Fraaije MW. (2006). Flavoprotein monooxygenase, a diverse class of oxidative biocatalysts. *Journal of Biotechnology*. 124:670-689.
- Bradski, G. (2000). *OpenCV_library*. Dr. Dobb's Journal of Software Tools.
- Briscoe AD, Chittka L. (2001) The Evolution of Color Vision in Insects. *Annu. Rev. Entomol.* 46: 471-510.
- Buck J, Case J. (2002) Physiological Links in Firefly Flash Code Evolution. *J. of Insect Behavior*. 15(1): 51-68.
- Buller AHR. (1924) The bioluminescence of *Panus stypticus*. Pp 357-431. In: *Researches on fungi*. Vol. III. Longsman, Green and Co., London.
- Camm EL, Towers GHN. (1973). Review article phenylalanine ammonia lyase. *Phytochemistry*. 12:961-973.
- Campagna C, Dignani J, Blackwell SB, Marin MR. (2001) Detecting bioluminescence with an irradiance time-depth recorder deployed on southern elephant seals. *Marine Mammal Science* 17:402–14.
- Campbell, A. K., and Herring, P. J. (1990). Imidazopyrazine bioluminescence in copepods and other marine organisms. *Mar. Biol.* 104: 219-225.
- Cantarel BL, Kork I, Robb SMC, Parra G, Ross E, Moore B, Holt C, Alvarado AS, Yandell M. (2008). MAKER: an easy-to-use annotation pipeline designed for emerging model organism genomes. *Genome Research*. 18:188-196.
- Capelari M, Desjardin DE, Perry BA, Asai T, Stevani CV. (2011) *Neonothopanus gardneri*: a new combination for a bioluminescent agaric from Brazil. *Mycologia*. 103(6): 1433-1440.
- Carlson AD, Copeland J. (1985) Flash Communication in Fireflies. *Quarterly Rev. of Biol.* 60(4):415-436.

- Castro AAJF, Martins FR, Fernandes AG (1998) The Woody Flora of Cerrado Vegetation in the state of Piaui, northeastern Brazil. *Edinb. J. Bot.* 55(3): 455-472.
- Cazares E, Trappe JM. (1994) Spore Dispersal of Ectomycorrhizal Fungi on a Glacier Forefront by Mammal Mycophagy. *Mycologia* 86(4): 507-510.
- Cock PJA, Antao T, Chang JT, Chapman BA, Cox Cj, Dalke A, Friedberg I, Hamelryck T, Kauff F, Wilczynski B, Hoon MJL. (2009) Biopython: freely available python tools for computational molecular biology and bioinformatics. *Bioinformatics.* 25(11):1422-1423.
- Colgan W, Claridge AW. (2002). Mycorrhizal effectiveness of Rhizopogon spores recovered from faecal pellets of small forest-dwelling mammals. *Mycol Res.* 106(3):314-320.
- Collins C, Keane TM, Turner DJ, O'Keeffe G, Fitzpatrick DA, Doyle S. (2013) Genomic and Proteomic dissection of the ubiquitous plant pathogen, *Armillaria mellea*: Toward a new infection model system. *Journal of Proteome research.* 2013(12):2552-2570.
- Conti E, Franks NP, Brick P. (1996). Crystal structure of firefly luciferase throws light on a superfamily of adenylate-forming enzymes. *Structure.* 4:287-298.
- Desjardin DE, Capelari M, Stevani CV.(2005) A new bioluminescent agaric from Sao Paulo, Brazil. *Fungal Diversity.* 18:9-14.
- Desjardin DE, Perry BA, Lodge DJ, Stevani CV, Nagasawa E. (2010) Luminescent *Mycena*: new and noteworthy species. *Mycologia.* 102: 459-477.
- Dubois R. (1887) Note sur la fonction photogénique chez la *Pholas dactylus*. *C R Soc Biol.* 39:564–566.
- Dunlap JS, Hastings JW. (1981) The biological clock in *Gonyaulax* controls luciferase activity by regulating turnover. *Journal of biological chemistry.* 256:10509-10518.
- Dunlap JC, Hastings JW, Shimomura O. (1980) Crossreactivity between the light-emitting systems of distantly related organisms: Novel type of light emitting compound. *Proc Natl Acad Sci.* 77(3):1394-1397.
- Eckstein JW, Hastings JW, Ghisla S. (1993) Mechanism of bacterial bioluminescence: 4a,5-dihydroflavin analogs as models for luciferase hydroperoxide intermediates and the effect of substituents at the 8-position of flavin on luciferase kinetics. *Biochemistry* 32, 404–411.
- Ehlting J, Buttner D, Wang Q, Douglas CJ, Somssich IE, Kombrink E. (1999) Three 4-coumarate:coenzyme A ligases in *Arabidopsis thaliana* represent two evolutionarily divergent classes in angiosperms. *The Plant Journal.* 19(1):9-20.
- Eisner T, Goetz MA, Hill DE, Smedley SR, Menwald J. (1997) Firefly “femmes fatales” acquire defensive steroids (lucibufagins) from their firefly prey. *PNAS.* 94: 9723-9728.
- Engbrecht J, Silverman M. (1984) Identification of genes and gene products necessary for bacterial bioluminescence. *PNAS.* 81:4154-4158.

- Esaias WE, Curl HC, Seliger HH. (1973) Action spectrum for a low intensity, rapid photoinhibition of mechanically stimuable bioluminescence in the marine dinoflagellates *Gonyaulax catenella*, *G. acatenella*, and *G. tamarensis*. *J. Cell. Physiol.* 82: 363–372.
- Fagan TF, et al. (1993). Cloning, expression and sequence analysis of cDNA for the Ca²⁺ binding photoprotein, mitrocomin. *FEBS Lett.* 333:301-305.
- Felsenstein J. (1989) PHYLIP - Phylogeny Inference Package (Version 3.2). *Cladistics.* 5: 164-166.
- Fleisher KJ, Case JF. (1995) Cephalopod Predation Facilitated by Dinoflagellate Luminescence. *Biol. Bull.* 189: 263-271.
- Franke R, Humphreys JM, Hemm MR, Denault JW, Ruegger MO, Cusumano JC, Chapple C. (2002) The Arabidopsis REF8 gene encodes the 3-hydroxylase of phenylpropanoid metabolism. *The Plant Journal.* 30(1):33-45.
- Fristrup KM, Harbison GR. (2002) How do Sperm Whales Catch Squids?. *Marine Mammal Science.* 18(1): 42-54.
- Galante TE, Horton TR, Swaney DP. (2011) 95% of basidiospores fall within 1m of the cap: a field-and modeling-based study. *Mycologia.* 103(6):1175-1183.
- Gatenby JB.(1959) Notes on the New Zealand Glow-worm, *Bolitophila* (*Arachnocampa*) *luminosa*. *Transactions of the Royal Society of New Zealand.* 87: 291-314.
- Gonzalez A, Hare JF, Eisner T. (1999) Chemical egg defense in *Photuris* firefly “femmes fatales”. *Chemoecology.* 9: 177-185.
- Gotz S, Garcia-Gomez JM, Terol J, Williams TD, Nagaraj SH, Nueda MJ, Robles M, Talon M, Dopazo J, Conesa A. (2008) High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids Resarch.* 38(10): 3420-3435.
- Goujon M, McWilliam H, Li W, Valentin F, Squizzato S, Paern J, Lopez R. (2010). A new bioinformatics analysis tools framework at EMBL-EBI. *Nucleic acids research.* 38:W695-W699
- Gouy M, Guindon S, Gascuel O. (2010) SeaView Version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol Biol Evol.* 27(2):221-224.
- Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Sdiconis X, Fan L, Raychowdhury R, Zeng Q, Chen Z, Maucili E, Hacohen N, Gnirke A, Rhind N, Palma F, Birren BW, Nusbaum C, Lindblad-Toh K, Friedman N, Regev A. (2011) Full-length transcriptome assembly from RNA-seq data without a reference genome. *Nature Biotechnology.* 29(7):644-652.
- Grigoriev IV, Nordberg H, Shabalov I, Aerts A, Cantor M, Goodstein D, Kuo A, Minovitsky S, Nikitin R, Ohm RA, Otilar R, Poliakov A, Ratnere I, Riley R, Smirnova T, Rokhsar D, Dubchak I. (2012) The genome portal of the department of energy joint genome institute. *Nucleic acids res.* 40:D26-32.

- Haddock SHD, Case JF. (1999) Bioluminescence spectra of shallow and deep-sea gelatinous zooplankton: ctenophores, medusae and siphonophores. *Marine Biology*. 133:571-581.
- Haddock SHD, Moline MA, Case JF. (2010) Bioluminescence in the Sea. *Annu. Rev. Mar. Sci.* 2: 443-493.
- Hall DT, et al. (1995) Detection of an oxygen atmosphere on Jupiter's moon Europa. *Nature*. 373:677-679
- Harvey EN (1952) *Bioluminescence*. Academic Press, New York.
- Harvey EN (1975). *A history of luminescence*. J.H Furst Company, Baltimore, Maryland.
- Hastings JW, Davenport D. (1957) The luminescence of the millipede, *Luminodesmus sequoiae*. *Biological Bulletin*. 113(1):120-128.
- Heimann K, Klerks PL, Hasenstein KH. (2009) Involvement of actin and microtubules in regulation of bioluminescence and translocation of chloroplasts in the dinoflagellate *Pyrocystis lunula*. *Botanica Marina*. 52:170-177.
- Herring PJ. (1994) Luminous Fungi. *Mycologist*. 8(4):181-183.
- Herring PJ, Cope C. (2005) Red bioluminescence in fishes: on the suborbital photophores of *Malacosteus*, *Pachystomias* and *Aristostomias*. *Marine Biology*. 148:383-394.
- Hertweck C (2009). The biosynthetic logic of polyketide diversity. *Angewandte chemie*. 48:4688-4716.
- Hoffmeister D, Keller NP. (2006) Natural products of filamentous fungi: enzymes, genes and their regulation. *Nat Prod Rep*. 24:393-416.
- Inouye, S., and Tsuji, F. I. (1993). Cloning and sequence analysis of cDNA for the Ca²⁺ activated photoprotein, clytin. *FEBS Lett*. 315: 343-346.
- Ivanauskas NM, Miashike RL, Godoy JRL, Souza FM, Kanashiro MM, Mattos IFA, Toniato MTZ, Franco GADC (2012). A vegetação do Parque Estadual Turístico do Alto Ribeira (PETAR), São Paulo, Brasil. *Biota Neotrop*. 12(1): 147-177.
- Jin H, Cominelli E, Bailey P, Parr A, Mehrtens F, Jones J, Tonelli C, Weisshaar B, Martin C. (2000) Transcriptional repression by AtMYB4 controls production of UV-protecting sunscreens in *Arabidopsis*. *EMBO*. 19(22):6150-6161.
- Jin J, Hughes KW, Petersen RH. (2001) Biogeographical patterns in *Panellus stypticus*. *Mycologia*. 93(2): 309-316.
- Johnson MEM. (1919) On the biology of *Panus stypticus*. *Trans Brit Mycol Soc*. 6:348-353.
- Johnson CN. (1996) Interactions between mammals and ectomycorrhizal fungi. 11(12):503-507.
- Keller NP, Turner G, Bennett JW(2005) Fungal secondary metabolism - from biochemistry to genomics. *Nature Reviews*. 3:937-947.

- Kent JW (2002) BLAT - the BLAST-like alignment tool. *Genome Research*. 12:656-664.
- Kim SH, Virmani D, Wake K, MacDonald K, Kronstad JW, Ellis BE. Cloning and disruption of a phenylalanine ammonia-lyase gene from *Ustilago maydis*. *Curr Genet*. 40:40-48.
- Kishi, Y., et al. (1966). Cypridinid bioluminescence I: structure of Cypridina luciferin. *Tetrahedron Lett.*, pp. 3427-3436.
- Koukol J and Conn EE. (1961) The metabolism of aromatic compounds in higher plants. *Journal of biological chemistry*. 236(10):2692-2698.
- Langmead B, Salzberg SL. (2012). Fast gapped-read alignment with bowtie 2. *Nature Methods*. 9(4):357-359.
- Li L, and Hastings JW. (1998) The structure and organization of the luciferase gene in the photosynthetic dinoflagellate *Gonyaulax polyedra*. *Plant Molecular biology*
- Lloyd JE. (1974). Bioluminescent communication between fungi and insects. *Fla. Entomol*. 57: 90
- Lloyd JE. (1984) Occurrence of Aggressive Mimicry in Fireflies. *Florida Entomologist*. 67(3): 368-376
- Lois R, Dietrich A, Hahlbrook K, Schulz W. (1989) A phenylalanine ammonia-lyase gene from parsley: structure, regulation and identification of elicitor and light responsive cis-acting elements. *EMBO*. 8(6):1641-1648.
- Macrae R. (1937) Interfertility phenomena of the American and European form of *Panus stipticus* (Bull.) Fries. *Nature* 139: 674.
- Malloch D, Blackwell M. (1992). Dispersal of Fungal Diaspores. In: *the Fungal Community*. Carrol GC, Wicklow DJ (eds.) Marcel Dekker, Inc. New York, New York.
- Marek P, Papaj D, Yeager J, Molina S, Moore W. (2011) Bioluminescent aposematism in millipedes. *Curr. Biol*. 21(18): 680-681.
- Martinez-Harms J, Vorobyev M, Schorn J, Shmida A, Keasar T, Homberg U, Schmeling F, Menzel R. (2012) Evidence of red sensitive photoreceptors in *Pygopleurus israelitus* (Glaphyridae: Coleoptera) and its implications for beetle pollination in the southeast Mediterranean. *J. Comp. Physiol A*. 198: 451-463.
- Matheny PB, Curtis JM, Hofstetter V, Aime MC, Moncalvo J, et al. (2006) Major clades of Agaricales: a multilocus phylogenetic overview. *Mycologia*. 98(6): 984-997.
- McAlpine D. (1900) Phosphorescent fungi in Australia. *Proc. Linn. Soc. New South Wales*. 25:548-558.
- Meighen EA. (1991) Molecular biology of bacterial bioluminescence. *Microbiol Rev*. 55:123-142.
- Meighen EA. (1994) Genetics of bacterial bioluminescence. *Annu Rev Genet*. 28:117-139

- Mendes LF, Bastos EL, Desjardin DE, Stevani CV. (2008) Influence of culture conditions on mycelial growth and bioluminescence of *Gerronema viridilucens*. *FEMS Microbiol Lett.* 282: 132-139.
- Mensing AF, Case JF. (1992) Dinoflagellate luminescence increases susceptibility of zooplankton to teleost predation. *Marine Biology.* 112: 207-210.
- Mihail JD, Bruhn JN. (2007) Dynamics of bioluminescence by *Armillaria gallica*, *A. mellea* and *A. tabescens*. *Mycologia.* 99(3):341-350.
- Morise, H., Shimomura, O., Johnson, F. H., and Winant, J. (1974). Intermolecular energy transfer in the bioluminescent system of *Aequorea*. *Biochemistry* 13: 2656-2662.
- Moriya Y, Itoh M, Okuda S, Yoshizawa AC, Kanehisa M. (2007) KAAS: an automatic genome annotation and pathway reconstruction server. *Nucleic Acids Research.* W182-W185.
- Nakajima Y, et al. (2004). cDNA cloning and characterization of a secreted luciferase from the luminous Japanese ostracod, *Cypridina noctiluca*. *Biosci. Biotechnol. Biochem.* 68: 565-570.
- Nakamura, H., Musicki, B., Kishi, Y., and Shimomura, O. (1988). Structure of the light emitter in krill *Euphausia pacifica*. *J. Am. Chem. Soc.* 110: 2683-2685.
- Nakamura H, Kishi Y, Shimomura O, Morse D, Hastings JW. (1989). Structure of dinoflagellate luciferin and its enzymatic and nonenzymatic air-oxidation products. *J. Am. Chem. Soc.* 111: 7607-7611.
- Nambudiri AMD, Vance CP, Towers GHN. (1973) Effect of Light on Enzymes of Phenylpropanoid Metabolism and Hispidin Biosynthesis in *Polyporus hispidus*. *Biochem J.* 134:891-897.
- Nambudiri AMD, Vance CP, Towers GHN. (1974) Styrylpyrone biosynthesis in *polyporus hispidus* II. Enzymatic hydrolysis of p-Coumaric acid and Bis-Noryangonin. *Biochemica et Biophysica Acta.* 343:148-155.
- Nelson DR et al. (1996) P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics.* 6:1-42
- O'kane DJ, Wilma LL, Porter D, Wampler O'Kane DJ, Lingle WL, Porter D, Wampler JE. (1990). Localization of Bioluminescent Tissues During Basidiocarp Development in *Panellus Stypticus*. *Mycologia.* 82(5): 595-606.
- Oliveira ML, Morato EF. (2000) Stingless bees (Hymenoptera, Meliponini) feeding on stinkhorn spores (Fungi, Phallales): robbery or dispersal?. *Rev Bras Zool.* 17: 881-884.
- Oliveira AG, Stevani CV. (2009) The enzymatic nature of fungal bioluminescence. *Photochem. Photobiol. Sci.* 8: 1416-1421.
- Oliveira AG, Desjardin DE, Perry BA, Stevani CV. (2012) Evidence that a single bioluminescent system is shared by all known bioluminescent fungal lineages. *Photochem. Photobiol.* 11: 848-852.

- Park IH, Jeon SY, Lee HJ, Kim SI, Song KS. (2004) A b-secretase (BACE1) inhibitor hispidin from the mycelial cultures of *Phellinus linteus*. *Planta Med.* 70:143–146
- Park J, Lee S, Choi J, Ahn K, Park B, Park J, Kang S, Lee YH. (2008) Fungal cytochrome P450 database. *BMC genomics.* 9:402.
- Perrin PW, Towers GHN. (1972) Hispidin biosynthesis in cultures of *Polyporus hispidus*. *Phytochemistry.* 12:589-592.
- Petersen RH, Bermudes D. (1992) *Panellus Stypticus*: Geographically Separated Interbreeding Populations. *Mycologia.* 84(2): 209-213.
- Purtov KV, Petushkov VN, Baranov MS, Mineev KS, Rodionova NS, Kaskova ZM, Tsarkova AS, Petunin AI, Bondar VS, Rodicheva EK, Medvedeva SE, Oba Yuichi, Yumiko Oba, Arseniev AS, Lukyanov S, Gitelson JI, Yampolsky IV. (2015). The chemical basis of fungal bioluminescence. *Angew Chem.* 127:8242-8246
- Ramsbottom J. (1953) *Mushrooms and toadstools*. Collins. London.
- Rawlings BJ. (1999) Biosynthesis of polyketides (other than actinomycete macrolides). *Nat Prod Rep.* 16:425-484.
- Rice P, Longden I, Bleasby A. (2000). EMBOSS: the European Molecular Biology Open Software Suite. *Trends in Genetics.* 16(6):276-277.
- Riley R, et al. (2014). Extensive sampling of basidiomycete genomes demonstrates inadequacy of the white-rot/brown-rot paradigm for wood decay fungi. *PNAS.* 111(27):9923-9928.
- Saia SEMG, Pessenda LCR, Gouveia SEM, Aravena R, Bendassolli JA (2008) Last glacial maximum (LGM) vegetation changes in the Atlantic Forest, southeastern Brazil. *Quaternary International.* 184: 195-201.
- Schoch G, Goepfert S, Morant M, Hehn A, Meyer D, Ullmann P, Werck-Reichert D. (2001). CYP98A3 from *Arabidopsis thaliana* Is a 3-Hydroxylase of Phenolic Esters, a Missing Link in the Phenylpropanoid Pathway*. *The Journal of Biological Chemistry.* 276(39):36566-36574.
- Schultz LW, Liu L, Cegielski M, Hastings JW. (2005) Crystal structure of a pH-regulated luciferase catalyzing the bioluminescent oxidation of an open tetrapyrrole. *PNAS.* 102:5:1378-1383.
- Seliger HH, and McElroy WD. (1964). The colors of firefly bioluminescence: enzyme configuration and species specificity. *Proc. Natl. Acad. Sci. USA* 52: 75-81.
- Sharpe ML, Dearden PK, Gimenez G, Krause KL. (2015). Comparative RNA seq analysis of the New Zealand glowworm *Arachnocampa luminosa* reveals bioluminescence-related genes. *BMC Genomics.* 16:825
- Shimomura O. (1981) A new type of ATP-activated bioluminescent system in the millipede *Luminodesmus sequoiae* *FEBS lett.* 128, 242-244

Shimomura O. (1984) Porphyrin chromophore in Luminodesmus photoprotein. *Comp Biochem Phys B*. 79:565-567.

Shimomura O. (2006) *Bioluminescence: Chemical Principles and Methods*. Wold Scientific Pub. Co. Singapore.

Shimomura O, Johnson FH. (1970). Mechanisms in the quantum yield of cypridina bioluminescence. *Photochem, Photobiol*. 12: 291-295.

Shimomura O, Johnson FH. (1971). Mechanism of the luminescent oxidation of Cypridina luciferin. *Biochem. Biophys. Res. Commun*. 44: 340-346.

Shimomura O, Goto T, Johnson FH. (1977). Source of oxygen in the CO₂ produced in the bioluminescent oxidation of firefly luciferin. *Proc. Natl. Acad. Sci. USA* 74: 2799-2802.

Shimomura O, Masugi T, Johnson FH, Haneda Y. (1978). Properties and Reaction Mechanism of the Bioluminescence System of the Deep-Sea Shrimp *Oplophorus gracilorostris*. *Biochemistry*. 17(6):994-998.

Sievers F, et al. (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology*. 7:539.

Silva JR, Amaral DT, Hastings JW, Wilson T, Viviani VR. (2014) A transcriptional and proteomic survey of *Arachnocampaluminosa* (Diptera: Keroplatidae) lanterns gives insights into the origin of bioluminescence from the Malpighian tubules in Diptera. *Luminescence* 30:996-1003.

Sivinski J. (1981) Arthropods Attracted to Luminous Fungi. *Psyche*. 88, 383-390.

Sivinski JM. (1998) Phototropism, Bioluminescence, and the Diptera. *Florida Entomologist*. 81(3): 282-292.

Slippers B, de Groot P, Wingfield MJ. (2012) *The Sirex Woodwasp and its Fungal Symbiont: Research and Management of a Worldwide Invasive Pest*. Springer.

Steidinger KA, Tangen K. (1997) Dinoflagellates. In: (C.R. Tomas, ed.) *Identifying marine phytoplankton*. Academic Press, San Diego. 387–584.

Stevani CV, Baader WJ. (1999) Chemiluminescent peroxy oxalate system. *Quimica Nova*. 22:715-723.

Stojanovic MN, Kishi Y. (1994). Dinoflagellate bioluminescence: the chromophore of dinoflagellate luciferin. *Tetrahedron Lett*. 35: 9343-9346.

Teutsch HG, HasenFratz MP, Lesot A, Stoltz C, Garnier JM, Jeltsch JM, Durst F, Werck-Reichhart D. (1993) Isolation and sequence of a cDNA encoding the Jerusalem artichoke cinnamate 4-hydroxylase, a major plant cytochrome P450 involved in the general phenylpropanoid pathway. 90:4102-4106.

- Thompson, E. M., Nagata, S., and Tsuji, F. I. (1989). Cloning and expression of cDNA for the luciferase from the marine ostracod *Vargulahlilgendorffii*. *Proc. Natl. Acad. Sci. USA* 86: 6567-6571.
- Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ, Pachter L. (2010). Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nature biotechnology*. 28(5):511-515.
- Tu SC (2007) Activity coupling and complex formation between bacterial luciferase and flavin reductases. *Photochem Photobiol Sci*. 7:183-188.
- Tuno N. (1998) Spore dispersal of *Diclyophora* fungi (Phallaceae) by flies. *Ecol Res*. 13: 7-15.
- Vliadi M, Iglesias-Rodriguez D. (2013) Understanding Bioluminescence in Dinoflagellates-How far have we come?. *Micororganisms* 1:3-25.
- Vance CP, Nambudiri AMD, Towers GHN. (1973). Cinnamate-4-hydroxylase in *Polyporus hispidus*. *Canadian Journal of Biochemistry*. 51(6):731-734.
- Vance CP, Tregunna EB, Nambudiri AMD, Towers GHN. (1973). Styrylpyrone biosynthesis in *polyporus hispidus*, I. action spectrum and photoregulation of pigment and enzyme formation. *Biochimica et Biophysica Acta*. 343:138-147.
- Vance CP, Nambudiri AMD, Wat CK, Towers GHN. (1975) Isolation and properties of hydroxycinnamate CoA ligase from *polyporus hispidus*. *Phytochemistry*. 14:967-969.
- Vanden Hoek TL, Erickson T, Hryhoczuk D, Narasimhan K. (1991) Jack O'Lantern Mushroom Poisoning. *Annals of Emergency Medicine*. 20(5): 559-561.
- Villa R, Willetts A. (1997) Oxidations by microbial NADH plus FMN-dependent luciferases from *Photobacterium phosphoreum* and *Vibrio fischeri*. *Journal of Molecular Catalysis B*. E2:193-197.
- Viviani V. (2002) The origin, diversity, and structure function relationships of insect luciferases. *Cell. Mol. Life Sci*. 59:1833–1850.
- Viviani VR, Bechara EJH, Ohmiya Y. (1999) Cloning, sequence analysis, and expression of active *Phrixothrix* railroad-worms luciferases: relationship between bioluminescence spectra and primary structures. *Biochemistry*. 38:8271–8279
- Viviani VR, Prado RA, Arnoldi FGC, Abdalla FC. (2009) An ancestral luciferase in the Malpighian tubules of a non-bioluminescent beetle. *Photochem. Photobiol. Sci*. 8:57–61.
- Viviani VR, Prado RA, Neves DR, Kato D, Barbosa JA. (2013) A Route from Darkness to Light: Emergence and Evolution of Luciferase Activity in AMP-CoA-Ligases Inferred from a Mealworm Luciferase-like Enzyme. *Biochemistry*. 52:3963-3973.

Watanabe H, Hastings HW. (1982) Specificities and properties of three reduced pyridine nucleotide-flavin mononucleotide reductases coupling to bacterial luciferase. *Molecular and Cellular Biochemistry*. 44:181-187.

Wawrzyn GT, Quin MB, Choudhary S, Lopez-Gallego F, Schmidt-Dannert C (2012) Draft Genome of *Omphalotus olearius* provides a predictive framework for sesquiterpenoid natural product biosynthesis in basidiomycota. *Chemistry & Biology*. 19: 772-783.

Webber JF. (2004) Experimental studies of factors influencing the transmission of Dutch elm disease. *Invest Agrar Sist Recur For*. 13(1):197-205.

Weijn A, Bastiaan-Net S, Wichers HJ, Mes JJ. (2013) Melanin biosynthesis pathway in *Agaricus bisporus* mushrooms. *Fungal Genetics and Biology*. 55:42-53.

Weng JK, Noel JP. (2012) Structure-Function analysis of plant type III polyketide synthases. *Methods in Enzymology*. 55:317-332.

White EH, McCapra F, Field G, McElroy WD (1961) The structure and synthesis of firefly luciferin. *Am Chem Soc*. 83:2402-2403.

Wilson T, Hastings JW (1998) Bioluminescence. *Annu Rev Dev Biol*. 14:197-230.

Wilson T, Hastings JW (2013) *Bioluminescence: Living light, lights for living*. Harvard University Press. Cambridge, Massachusetts.

Wood KV, De Wet JR, Dewji N, DeLuca M. (1984). Synthesis of active firefly luciferase by in vitro translation of RNA obtained from adult lanterns. *Biochem. Biophys. Res. Commun*. 124: 592-596.

Wood KV, Lam YA, Seliger HH, and McElroy WD. (1989). Complementary DNA coding click beetle luciferase can elicit bioluminescence of different colors. *Science* 244: 700-702.

Zdobnov EM, Apweiler R. (2001). InterProScan - and integration platform for the signature-recognition methods in InterPro. *Bioinformatics Applications Note*. 17(9):847-848.

Zenno S, Saigo K (1994) Identification of the Genes Encoding NAD(P)H-Flavin Oxidoreductases That Are Similar in Sequence to *Escherichia coli* Fre in Four Species of Luminous Bacteria: *Photobacterium luminescens*, *Vibrio fischeri*, *Vibrio harveyi*, and *Vibrio orientalis*. *Journal of Bacteriology*. 176(12):3544-3551.

Zhao Y, Xi Q, Xu Q, Meihong H, Ding J, Dai Y, Keller N, Zheng W. (2015) Correlation of nitric oxide produced by an inducible nitric oxide synthase-like protein with enhanced expression of the phenylpropanoid pathway in *Inonotus obliquus* cocultured with *Phellinus morii*. *Appl Microbiol Biotechnol*. 99:4361-4372.

Appendix List

Appendix A: P450omes

Appendix B: Bioinformatics databases (DVD)

145

TU p450ome similarity

LE p450ome similarity