

COMPARATIVE ANALYSIS OF LUMINESCENCE INTENSITY OF SINGLE
SPORE ISOLATES FROM THE NATURALLY BIOLUMINESCENT FUNGUS
ARMILLARIA MELLEA (AGARICALES, PHYSALACRIACEAE)

by

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A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of
Master of Science in Biology

Middle Tennessee State University
May 2018

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ACKNOWLEDGEMENTS

I would like to thank Dr. Sarah Bergemann for her patience and guidance throughout this project and the many, many thesis drafts. I would like to thank Dr. J. Brian Robertson for all his advice, biotechnology skills and encouragement throughout the entirety of my time here at MTSU. And thank you to Dr. Kendra Baumgartner for not only providing the environmental isolate Eldo17, but also the non-luminescent transformant, without which none of this research would have been possible.

Thank you to the MTSU Biology Department for partial funding my research through scholarships (Kurt E. Blum Botany Research Scholarship and Mary C. Dunn Graduate Scholarship), travel funds to attend conferences and funding from the Faculty Research and Creative Activity Committee (FRCAC) awarded to Dr. J. Brian Robertson.

I would also like to thank my fellow graduate students in the Biology Department for their never-ending support, idea bouncing and comradery. I would never have been able to get through this program without them.

Lastly, I would like to thank my parents Andrew and Judith Poole for their encouragement and support and my son Bruce for being my pillar through thick and thin.

ABSTRACT

Bioluminescence is the production and emission of light by a living organism which requires the oxidation of a luciferin substrate by a luciferase enzyme or photoprotein to produce light. Nearly all the described fungi that bioluminesce are mushroom-forming, saprotrophic species belonging to four, distantly related lineages in the Agaricales (Basidiomycota). Here, I conduct comparative studies to examine the variation in luminescence intensity of single spore isolates (SSIs) of the bioluminescent fungus, *Armillaria mellea*. A diploid isolate of *A. mellea* was inoculated on sawdust, rice and tomato media and incubated to induce formation of basidiomata *in vitro*.

Luminescence intensity of single spore isolates (SSIs) from haploid basidiospores were obtained and classified as three phenotypes (bright, dim and intermediate) based on their luminescence intensity. Three bright (>1000 AU), four intermediate (200-999 AU) and three dim SSIs (<200 AU) were selected for pairings in the following combinations: i) bright × bright; ii) intermediate × intermediate and; iii) dim × dim luminescence intensity. In addition, dim and bright luminescent isolates were paired with a low luminescent transformant obtained from the USDA-ARS. The frequency and luminescence intensity of haploid SSIs ($n = 241$) obtained from matings from five compatible mycelial pairings that produced basidiomata and matured to generate viable basidiospores [bright × bright ($n = 2$), intermediate × intermediate ($n = 1$), dim × transformant ($n = 2$)] and differences in luminescence intensity were compared at 14, 23 and 32 days post inoculation (dpi). Mean luminescence intensity of SSIs obtained from bright × bright matings were greater than SSIs obtained from intermediate × intermediate

and dim × transformant matings. The differences in mean luminescence intensity suggests that the expression of bioluminescence is a heritable trait and exhibits a continuous distribution in luminescence intensity. In addition, matings of bright × bright isolates produced a higher frequency of SSIs with bright luminescence intensity (92%); however, 50% of SSIs obtained from the intermediate × intermediate cross and 21% of SSIs generated from the dim × transformant crosses exhibited bright luminescence intensity. This suggests that bioluminescence is a complex trait involving the interactions of multiple genes.

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CHAPTER 1. INTRODUCTION

Bioluminescence is the production and emission of light by a living organism. Bioluminescence occurs when a substrate (a luciferin) is catalyzed by an enzyme (a luciferase or photoprotein) to react with molecular oxygen forming the excited product (oxyluciferin) and giving off visible light (Shimomura 1989; Wilson and Hastings 1998). Bioluminescence has likely evolved at least 40 times in a diverse array of eukaryotes (e.g. shrimp, beetles, dinoflagellates, cnidarians, fungi and algae) as well as bacteria (Harvey 1952; Nealson and Hastings 1979; Haddock et al., 2010). Consequently, the bioluminescence systems of these organisms exhibit vast differences in chemistries of substrates and products and is not evolutionarily conserved across all species (Hastings 1996; Oliveira and Stevani 2009). Of these organisms, fungi that exhibit bioluminescence are found in four lineages (Oliveira et al., 2012).

Nearly all the 80 described species of bioluminescent fungi are mushroom-forming, saprotrophic species belonging to four, distantly related lineages in the Agaricales (Basidiomycota) which include the families Physalacriaceae, Omphalotaceae, Mycenaceae and a fourth unnamed lineage, *Lucentipes* (Desjardin et al., 2008; Stevani et al., 2013; Mihail 2015; Kaskova et al., 2017). A recent study demonstrated cross-reactivity across species from different families indicating fungi share a common bioluminescent chemistry that is conserved (Oliveira et al., 2012). Bioluminescent fungi may fulfill a variety of ecological functions including attraction of animals for spore dispersal or deterrence of predators by warning nocturnal fungivores (reviewed by Desjardin et al., 2008). In addition to these functions, this mechanism may not be

adaptive in fungi but may provide protection against the harmful effects of reactive oxygen during cellular respiration or lignin degradation (Desjardin et al., 2008; Baumgartner et al., 2011).

Among the luminescent fungi that are both saprotrophs and pathogens (facultative necrotrophs) of plant species are members of the genus *Armillaria* (Physalacriaceae, Agaricales) (Desjardin et al., 2008; Baumgartner et al., 2011; Oliveira et al., 2012; Stevani et al., 2013). To date, bioluminescence has been confirmed for ten *Armillaria* species distributed globally (Desjardin et al., 2008; Mihail 2015). Bioluminescence is not uniformly distributed in mycelia of all agaric fungi that luminesce (for example, the vegetative mycelia and the basidiomata may be luminescent in some species but not in others) (Desjardin et al., 2008). In *Armillaria*, only the vegetative mycelia (including young rhizomorphs which are hyphal aggregates that are involved in resource capture) are bioluminescent (Desjardin et al., 2008; Baumgartner et al., 2011; Mihail 2015) and no diurnal or seasonal oscillations of luminescence were confirmed for *Armillaria* despite earlier reports of these fluctuations (Mihail and Bruhn 2007; Baumgartner et al., 2011). *Armillaria* spp. often exhibit consistent luminescence intensity when cultured under controlled laboratory conditions, but luminescence intensity may vary with environmental stimuli (e.g., pH or temperature) (Coblentz and Hughes 1926; Bermudes et al., 1990; Weitz et al., 2001; Desjardin et al., 2008).

The hyphae that germinate from basidiospores of *Armillaria* are haploid and mating between compatible hyphae (single spore isolates or SSIs) is required to produce a fertile mycelium (Ullrich and Anderson 1978). When compatible SSIs (which exhibit a

fluffy morphology) are paired in culture, the fertile mycelia develop into a crustose mycelium whereas incompatible pairings retain the fluffy morphology due to an abundance of aerial mycelia (Anderson and Ullrich 1978). Mating of haploid SSIs of *A. mellea* are compatible in a pattern typical of bifactorial heterothallism (which requires two different idiomorphs or alleles at two mating loci for compatible pairings) and as a consequence, compatibility of mating between siblings is reduced to 25% (Ullrich and Anderson 1978; Moore and Frazer 2002). *Armillaria* spp. are unusual in that the fertile mycelium is diploid, rather than dikaryotic, as is the case for most basidiomycetes (Anderson and Kohn 2007). The fertile, diploid mycelia may also be reliably cultured *in vitro* to form basidiomata that release haploid basidiospores by forcible discharge from the spore-bearing structures (basidia) (Ford et al., 2015). The production of viable basidiospores from basidiomata of *A. mellea* allows for the propagation of SSIs.

Variation in the luminescence intensity of the mycelia was reported in several bioluminescent agarics (Lingle et al., 1992; Petersen and Bermudes 1992). For example, Berliner (1963) noted that the fertile mycelia of *A. mellea* consistently exhibited greater luminescence intensity than SSIs. Similar observations were noted for bioluminescent agarics including *Panellus stipticus*, *Lampteromyces japonicas* and *Mycena haematopus* in which the fertile mycelia obtained from matings of SSIs exhibited greater luminescence intensity than haploid mycelia (Bermudes et al., 1992; Petersen and Bermudes 1992). In contrast to these results, Mihail (2015) did not observe consistent differences in luminescence intensity of haploids and diploids of *Armillaria* spp.;

however, large and ephemeral changes in luminescence intensity were observed for the majority of species in the study.

Most of the research on the bioluminescence of *Armillaria* has focused on comparing luminescence intensity of species, comparing differences in luminescence intensity of haploids and diploids and how culture environment affects luminescence (Mihail and Bruhn 2007; Mihail 2013; Mihail 2015). At present, there are no studies of the inheritance of the bioluminescence trait in *Armillaria*. Based on the observation that diploids of *Armillaria* luminesce at a greater intensity than haploids, I propose that allelic complementation in diploids may mask the expression of this trait and as such, inheritance of this trait is more readily observed in haploid progeny. Additionally, haploids produced by mating of parental phenotypes with variable luminescence intensity will luminesce at an intensity to that observed in the parents. I also expect that the trait is polygenic (gene expression is controlled by multiple genes) and therefore, haploid progeny should exhibit a continuous frequency of bioluminescence intensity. These hypotheses could be tested by measuring the luminescence intensity of the haploid progeny and comparing the intensity of luminescence of progeny mated with variable luminescence intensity (bright, intermediate and dim). Therefore, the objectives of this research are to utilize *in vitro* fruiting, mating of isolates with variable luminescence intensity and compare the luminescence intensity and frequency of haploid progeny of *A. mellea* with varying luminescent intensities.

CHAPTER 2. MATERIALS AND METHODS

2.1. Fruiting of *Armillaria mellea*

To analyze the luminescence intensity of haploid mycelia of *A. mellea*, a collection of basidiospores was produced by *in vitro* fruiting of a fertile, diploid isolate obtained from the USDA-ARS, Davis, CA (Eldo17) (Ford et al., 2015). Mycelia of Eldo17 were cultured on potato dextrose agar (PDA) at 25°C for 2-3 weeks in darkness, plugs from the growing mycelia (ca. 5 mm in diameter) were excised using a cork borer and used to inoculate sawdust, rice and tomato (SRT) media (Ford et al., 2015) in 500 mL Magenta jars (Magenta LLC, Chicago, IL). SRT media was prepared by adding 50 mL deionized water to 12 g rice, 6 g of red oak sawdust and a ca. one cm layer of homogenized tomato (prepared by blending) to the top of the media. After the media was autoclaved, mycelial plugs were added to the cultures and were incubated in darkness at 25°C for three weeks. Once the cultures were colonized (rhizomorphs were observed colonizing the media), the cultures were transferred to a growth chamber with a diurnal cycle under white fluorescent lighting ($125 \mu\text{mol m}^{-2} \text{s}^{-1}$) (12 h light, 12 h dark) at 25°C for six weeks. After six weeks of incubation, cultures were transferred to an environmental chamber with humidity set to 70% and white fluorescent lighting ($5 \mu\text{mol m}^{-2} \text{s}^{-1}$) (10 h light, 14 h dark) to induce formation of basidiomata (Ford et al., 2015).

2.2. Basidiospore collection

Basidiospores were collected from mature basidiomata obtained by *in vitro* fruiting of *A. mellea*. The pilei of basidiomata were excised and placed in sterile 25 mL petri plates and incubated overnight in a growth room at 15°C with humidity of 70%.

After 12-24 h, petri plates were flooded with ca. 750 μ L sterile deionized water and gently agitated to remove the basidiospores from the plate. Basidiospores were collected by repeat pipetting of the suspension and stored at 4°C for 1-21 days.

2.3. *Single spore isolations*

Basidiospore suspensions of *A. mellea* were diluted 1:1000 and plated on 25 mL 1% malt extract agar (MEA) plates. Basidiospores were incubated at 25°C in darkness until germination was observed (3-5 days). Mycelia germinating from SSIs were excised from the media with a scalpel and transferred to each MEA plate and incubated in darkness at 25°C.

2.4. *Luminescence intensity of SSIs*

The luminescence intensity of *A. mellea* SSIs was measured using mycelia cultured on 1% MEA plates. To quantify, the BioRad ChemiDoc MP system was used to screen the cultures grown on MEA plates from 14-32 days post inoculation (dpi). BioRad imager bioluminescent measurements were collected at 60 s over 5 min intervals and compiled as a composite image (Figure 1). Mean luminescence intensity was quantified from the composite image using IMAGEJ software and expressed in arbitrary units (AU) (Schneider et al., 2012). Additionally, a bright-field photograph (0.1 s exposure time) was taken of the isolates to maintain records (Figure 2). Luminescence intensity of SSIs was also measured using a luminometer (Titertek-Berthold, Pforzheim, Germany) to obtain the rate at which light is emitted and expressed in relative light units per second (RLU/s).

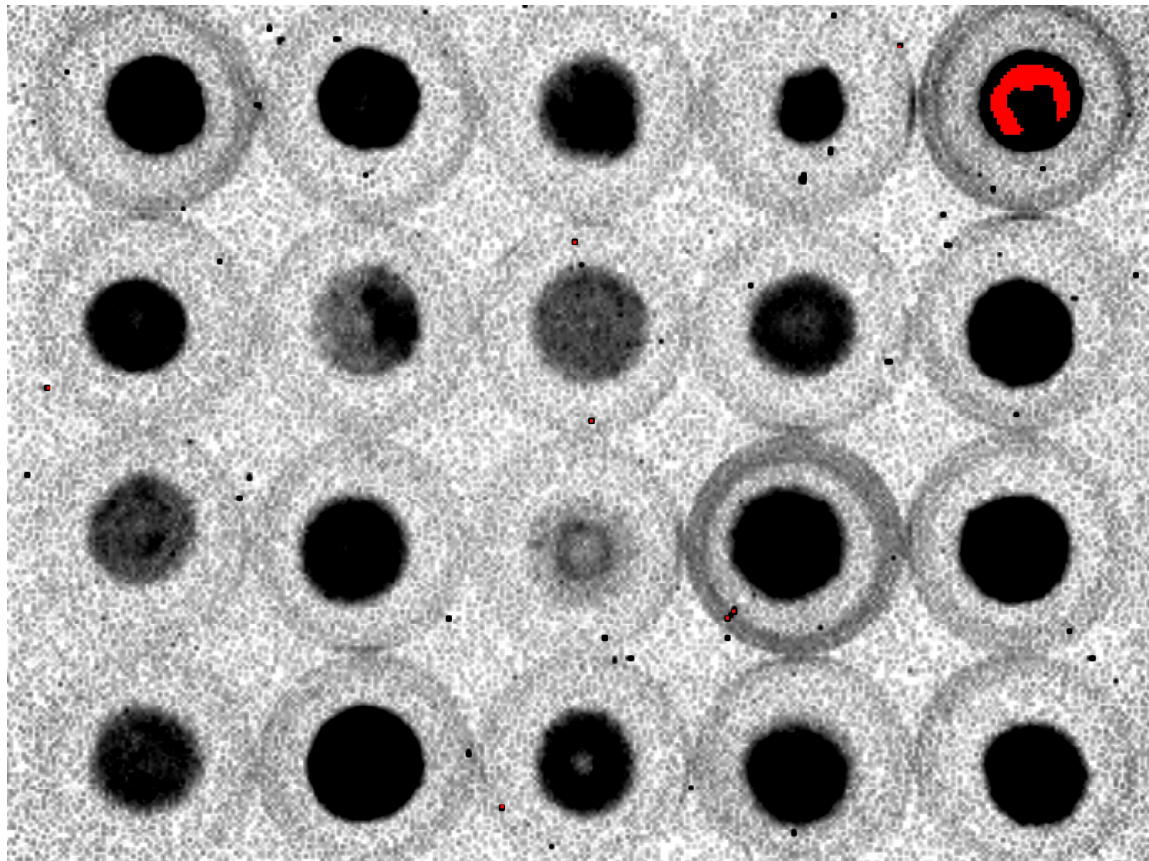


Figure 1. An example of a composite image of *Armillaria mellea* single spore isolates taken from the BioRad imager used for bioluminescence measurements (AU) at 32 days post inoculation. The shaded red portion indicates that the luminescence intensity of the isolate has reached the maxima of the imager.

2.5. Characterization of luminescence intensity of SSIs

SSIs used in the matings were assigned to phenotypes based on the mean luminescence intensity of all isolates measured at 14-32 dpi: i) bright ($\geq 1,000$ AUs); ii) intermediate (200 - 999 AUs) and; iii) dim (< 200 AUs). Eighteen possible pairings were generated from 10 selected SSIs based on their luminescence intensity (3 bright, 4 intermediate and 3 dim) for mating tests (Table 1). In addition, the SSIs with bright and dim luminescent phenotypes were paired with a transformant of *A. mellea* that exhibited low luminescent intensity to observe the luminescence intensity of the haploid progeny. The transformant was generated by *Agrobacterium tumefaciens*-mediated random insertional mutagenesis as previously described (Baumgartner et al., 2010). The genetic basis of the transformation of *A. mellea* was performed by the transfer of a large tumor-inducing plasmid (T-DNA) from *A. tumefaciens* and integration into the fungal nuclear genome of *A. mellea* (Baumgartner et al., 2010). The insertion of the T-DNA in the fungal genome of *A. mellea* resulted in a transformant that showed low levels of bioluminescence consistent with the intensity of dim phenotypes. Pairings between the low luminescent transformant (Table 1) were performed with dim and bright SSIs.

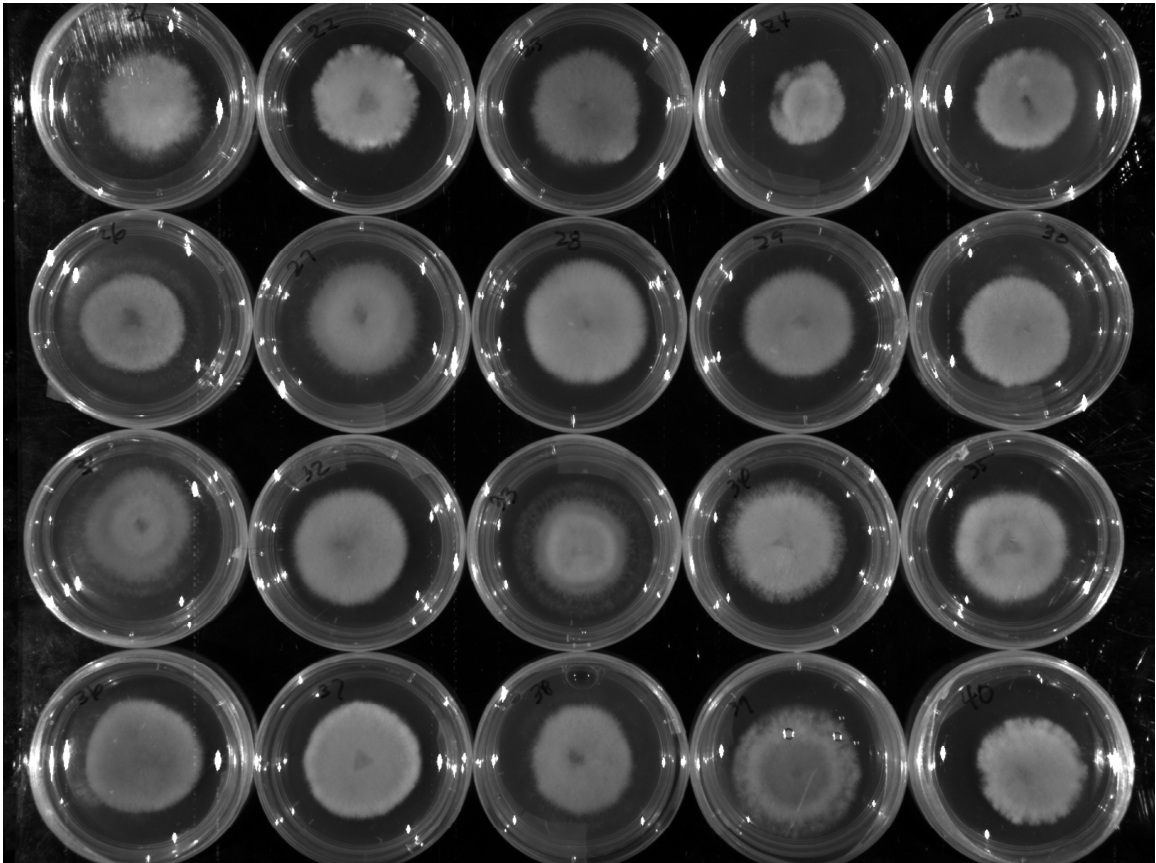


Figure 2. An example of a bright-field composite image of *Armillaria mellea* single spore isolates taken from the BioRad imager at 32 days post inoculation.

Table 1. Mean luminescence intensity of single spore isolates of *Armillaria mellea* used for matings at 14-32 days post inoculation (AU) and luminescence at 32 days post inoculation (RLU/s).

Luminescence intensity phenotype	Isolate	Mean luminescence intensity (AU)	Luminescence intensity (RLU/s)
bright	Eldo17ss72	2364	540440
	Eldo17ss88	2329	485380
	Eldo17ss109	1456	292500
intermediate	Eldo17ss28	782	214770
	Eldo17ss35	783	345030
	Eldo17ss39	826	174970
	Eldo17ss87	915	196700
dim	Eldo17ss50	174	573
	Eldo17ss95	192	1900
	Eldo17ss102	172	629
transformant	Son202pBGgHg1222PT25	-	655

2.6. Mating of SSIs based on luminescent phenotype

Bright, intermediate and dim SSIs used for pairings were cultured on PDA for two weeks at 25°C in darkness. After 2 weeks, pairings of SSIs were plated in duplicate on Shaw and Roth agar (SRA) (Shaw and Roth 1976) by placing 4 mm plugs of each SSI approximately 2 cm apart (Anderson and Ullrich 1979). Pairings of SSIs were conducted using the following combinations: i) bright × bright ($n = 3$); ii) intermediate × intermediate ($n = 6$); iii) dim × dim ($n = 3$); iv) bright × transformant ($n = 3$) and; v) dim × transformant ($n = 3$) (Table 2). Pairings of SSIs were incubated at room temperature in the dark at 25°C and routinely monitored for 2-3 weeks to assess mating compatibility. Pairings were scored as compatible (crustose) or incompatible (fluffy) based on the morphology of the mycelia (Anderson and Ullrich 1982).

2.7. Statistical analysis

A two-way mixed-model repeated measures ANOVA with one factor (luminescent phenotype of parental SSIs) was used to compare luminescence intensity at 14, 23 and 32 dpi of SSIs (O'Brien and Kaiser 1985). Based on the variation in SSIs generated from the bright × bright, intermediate × intermediate and dim × transformant matings, a mixed-models approach with an unstructured covariance matrix was used. Since equal variance was not assumed due to the large variance in luminescence intensity measured with the imager (AU) and luminometer (RLU/s), \log_{10} transformations were applied. Šidák pairwise comparisons were performed to determine whether luminescence intensity differed among the SSIs generated from all successful matings [bright × bright ($n = 2$), intermediate × intermediate ($n = 1$) and dim × transformant ($n = 2$)] and for each

time interval (14, 23 and 32 dpi) (Šidák 1967; O'Brien and Kaiser 1985). A contingency table analysis using a chi-square goodness of fit test was also performed to test whether luminescence intensity of SSIs from each parental mating were associated with luminescence intensity of parental SSIs (Pearson 1900). All statistical tests were performed in SPSS (IBM Corp. 2012) and considered significant at $P < 0.05$.

Table 2. Single spore isolates of *Armillaria mellea* used in pairings to demonstrate mating compatibility, formation of basidiomata and the viability of basidiospores.

Parental SSIs	Parental SSI luminescent phenotype	Mating compatibility (+) or incompatibility (-)	Basidiomata produced (+/-)	Basidiomata aborted (+)	Basidiospores viable (+) or inviable (-)
Eldo17ss72 × Eldo17ss109	bright × bright	+	+		+
Eldo17ss72 × Eldo17ss88	bright × bright	-	-		
Eldo17ss88 × Eldo17ss109	bright × bright	+	+		+
Eldo17ss28 × Eldo17ss35	intermediate × intermediate	-	-		
Eldo17ss28 × Eldo17ss39	intermediate × intermediate	-	-		
Eldo17ss28 × Eldo17ss87	intermediate × intermediate	-	-		
Eldo17ss35 × Eldo17ss39	intermediate × intermediate	+	+		+
Eldo17ss35 × Eldo17ss87	intermediate × intermediate	-	-		
Eldo17ss39 × Eldo17ss87	intermediate × intermediate	+	+		-
Eldo17ss50 × Eldo17ss95	dim × dim	-	-		
Eldo17ss50 × Eldo17ss102	dim × dim	-	-		
Eldo17ss95 × Eldo17ss102	dim × dim	-	-		
Eldo17ss72 × Son202pBGgHg1222PT25	bright × dim transformant	+	+	+	
Eldo17ss88 × Son202pBGgHg1222PT25	bright × dim transformant	-	-		
Eldo17ss109 × Son202pBGgHg1222PT25	bright × dim transformant	-	-		
Eldo17ss50 × Son202pBGgHg1222PT25	dim × dim transformant	+	+	+	
Eldo17ss95 × Son202pBGgHg1222PT25	dim × dim transformant	+	+		+
Eldo17ss102 × Son202pBGgHg1222PT25	dim × dim transformant	+	+		+

CHAPTER 3. RESULTS

Luminescence intensity was measured from SSIs produced from basidiomata of five compatible pairings (Table 2). Of the 18 combinations of paired SSIs, 67% of the bright × bright, 33% of the intermediate × intermediate and 0% of the dim × dim were compatible and produced fertile mycelia (Table 2). Of the successful pairings with the transformant, 66% (bright × transformant and dim × transformant pairings) produced basidiomata; however, one bright × transformant and one dim × transformant pairing did not mature as evidenced by primordia that were aborted (Table 2). One of the pairings of intermediate × intermediate resulted in a successful mating but basidiomata failed to produce viable basidiospores (Table 2). SSIs used in this study were generated from the successful matings of two bright × bright, one intermediate × intermediate and two dim × transformant SSIs (Table 2).

Luminescence intensity was estimated for SSIs from bright × bright ($n = 95$), intermediate × intermediate ($n = 50$) and dim × transformant matings ($n = 96$). Analyses were conducted on measurements collected from the imager (AU) and luminometer (RLU/s), both of which exhibited similar results in comparisons of mean luminescence intensity. A significant interaction was found for mean luminescence intensity of SSIs from parental phenotypes and time ($P < 0.05$) (Table 3; Figure 3). This result showed that luminescence intensity increased over time (from 14-32 dpi) and differed with respect to luminescence intensity depending on the phenotype of parental isolate mated (Table 3; Figure 3). The SSIs from bright × bright matings exhibited a greater mean luminescence intensity [2.5 – 4.5× difference in magnitude of luminescence intensity

(AU) and 6.0 – 9.0× difference in magnitude of luminescence intensity (RLU/s)] at 23 and 32 dpi than the SSIs produced from the intermediate × intermediate and dim × transformant matings (Figure 3). Furthermore, mean luminescence intensity of SSIs differed for all SSIs analyzed showing increases over time with the highest luminescence intensity at 32 dpi as compared to 14 and 23 dpi (Table 3; Figure 3).

Table 3. The results of the mixed-model two-way repeated measures analysis of variance (ANOVA) of luminescence intensity of single spore isolates of *Armillaria mellea*. *F* values and the level of significance (* = $P < 0.05$) are shown for all matings (bright × bright, intermediate × intermediate and dim × transformant) and the time intervals measured (14, 23 and 32 days post inoculation). Measurements obtained from the luminometer are reported in relative light units per second (RLU/s) and from the imager in arbitrary units (AU).

Factors	Time								
	14		23		32				
<i>Light Emitted (RLU/s)</i>	F	P	F	P	F	P	F	P	
Parental Mating	178.33	*	85.731	*	169.7	*	141.7	*	
Time	380.86	*							
Mating * Time	30.989	*							
<i>Mean Luminescence (AU)</i>									
Parental Mating	103.15	*	6.593	*	103.86	*	117.2	*	
Time	521.5	*							
Mating * Time	75.067	*							
			Luminescent Phenotype of Parental Matings						
Factors	Bright		Intermediate		Dim				
<i>Light Emitted (RLU/s)</i>	F	P	F	P	F	P	F	P	
Time	232.47	*	155.1	*	39.2	*		*	
<i>Mean Luminescence (AU)</i>									
Time	504.16		153.4		35.52				

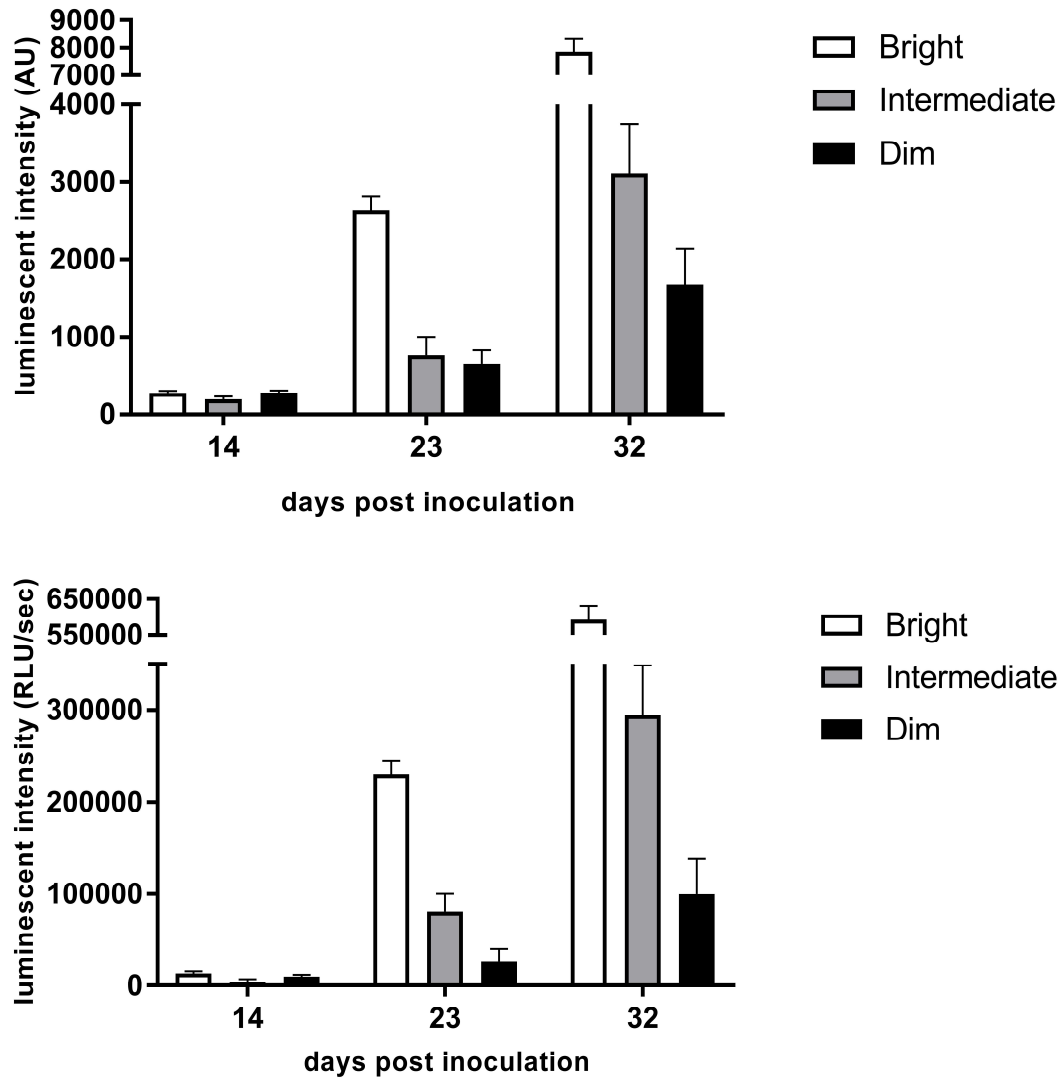


Figure 3. Mean luminescence intensities (+1 SE) of single spore isolates obtained from the matings of single spore isolates (bright \times bright, intermediate \times intermediate and dim \times transformant) across the time intervals measured (14, 23 and 32 days post inoculation). Hash marks were used for visualizing the mean luminescence intensity across all time intervals.

The frequency of isolates exhibiting bright, intermediate and dim phenotypes for each parental mating was also estimated. Analyses were conducted based on the initial observations in luminescence intensity of the parental isolates (bright = $\geq 1,000$ AU, intermediate = 200 - 999 AU and dim = < 200 AU). However, the frequency distribution was generated with increments of 200 AU to observe the distribution of frequencies of luminescence intensity of SSIs at a higher resolution (Figure 4). The mycelia of SSIs generated from the bright \times bright matings had a higher frequency of isolates with bright luminescence intensity (92%) with only 8% of isolates exhibiting intermediate luminescence intensity (Table 4; Figure 4). Fifty percent of the SSIs from intermediate \times intermediate matings exhibited an intermediate luminescence intensity. In contrast, the dim \times transformant matings produced a higher variance with 44% dim, 35% intermediate and 21% bright. The results of the chi-square contingency table analyses indicated a significant association of luminescence intensity of SSIs and parental phenotypes for all tests performed (Table 4; $P < 0.05$).

Table 4. The frequency of *Armillaria mellea* single spore isolates obtained from matings (bright × bright, intermediate × intermediate and dim × transformant) based on the mean luminescence intensity in arbitrary units (AU). The frequency of the observed and expected values (in parentheses) are shown for each category of luminescence intensity and the results of the test statistic (χ^2 and probability (P) for all tests comparing the observed and expected frequencies of bright, intermediate and dim progeny.

Mean Luminescence intensity	Bright	Intermediate	Dim
200	0 (16.5)	0 (9)	42 (16.5)
200-399	1 (9)	2 (5)	20 (9)
400-599	1 (6.0)	6 (3.0)	8 (6.0)
600-799	4 (6.5)	10 (4)	3 (6.5)
800-999	2 (5)	7 (2.0)	3 (5)
≥1000	87 (52.5)	25 (27)	20 (52.5)
χ^2	52.09 ($P < 0.05$)	17.79 ($P < 0.05$)	61.64 ($P < 0.05$)

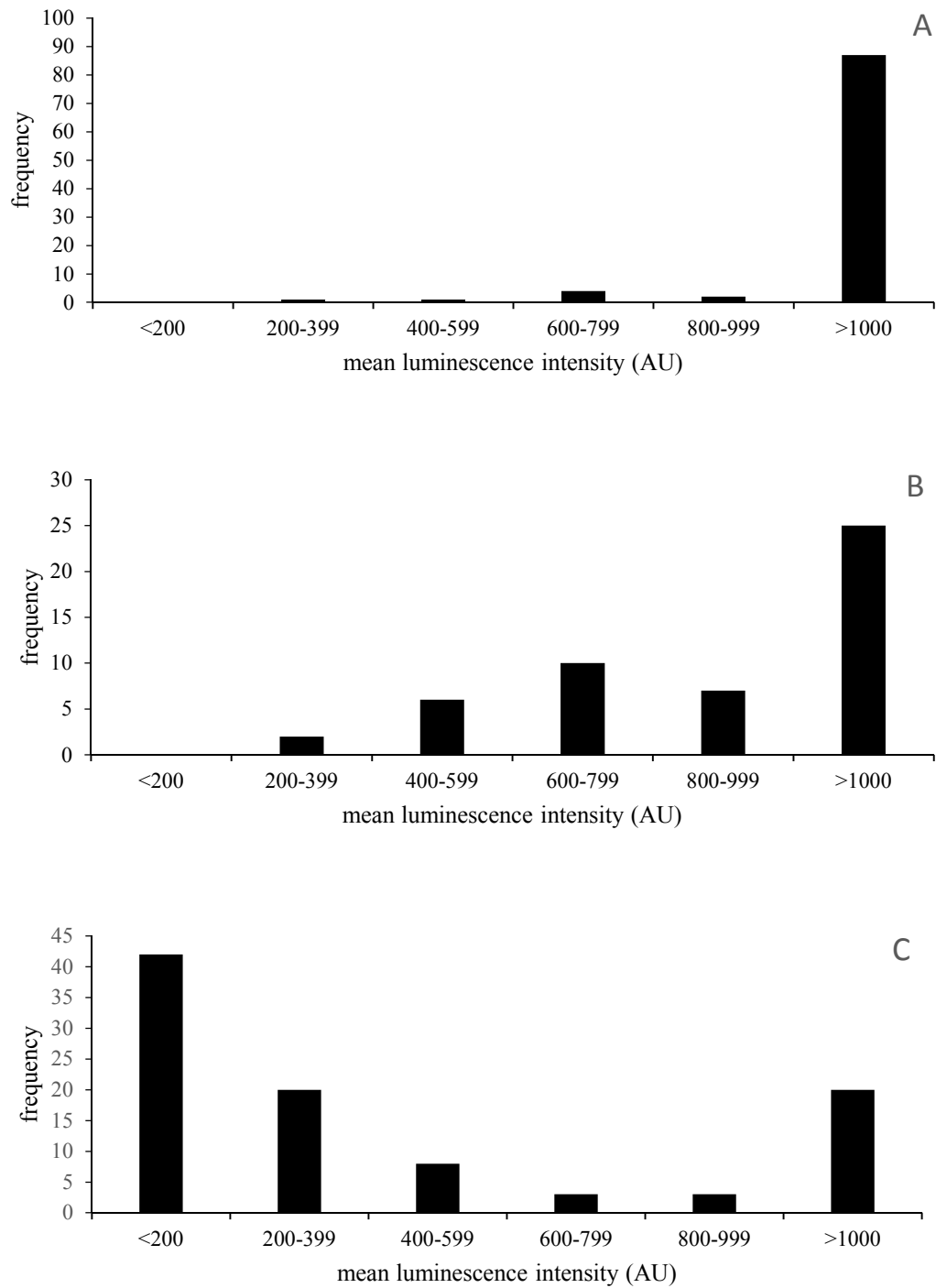


Figure 4. Frequency of luminescence intensity of single spore isolates of *Armillaria mellea* obtained from matings (bright × bright = A, intermediate × intermediate = B and dim × transformant = C).

CHAPTER 4. DISCUSSION

Bioluminescence is a notable phenomenon in *Armillaria* and the results of this study confirm that *A. mellea* is among one of the bioluminescent species exhibiting variation in luminescence intensity (Mihail and Bruhn 2007; Mihail 2015). The present study extends the previous observations of bioluminescence of *A. mellea* by comparing luminescence intensity of SSIs generated from mating that exhibit varying luminescence intensities. The mean luminescence intensity was higher for SSIs from matings between parental isolates exhibiting bright luminescence intensity than for matings between SSIs with lower intensity. Furthermore, the frequency distribution of luminescence is continuous. These results suggest that bioluminescence is heritable, and that gene expression is polygenic.

Given the variation observed of *Armillaria* luminescence, this mechanism may also be constrained by sensitivity to periodic environmental stimuli (reviewed by Stevani et al., 2013). In this study, observations of luminescence intensity of SSIs were performed by culturing on MEA agar plates which contains digests of peptones from animal tissues to provide an ample source of amino acids and other nitrogenous compounds for the growth of *Armillaria*. One of the advantages of using agar media is that each SSI is treated as a replicate grown under similar conditions and this decreases the experimental variability associated with mycelia from different culture environments. Temperature and pH (which were not measured in this study) are well known to affect luminescence intensity (Weitz et al., 2001). In these experiments, environmental variables were not measured *in situ*, but all replicates were cultured under similar

conditions and, as a consequence, is considered reliable for estimating luminescence intensity as it reflects the consistent culture environment.

These results show that luminescence intensity varies considerably under uniform culture environments (reviewed by Desjardin et al., 2008). Mihail and Bruhn (2007) measured luminescence intensity of mycelia from three *Armillaria* spp. cultured on different media and found interspecific differences in luminescence intensity of *Armillaria* spp. that also differed with respect to the media used for culturing (Mihail and Bruhn 2007). Mihail (2015) also reported large, ephemeral differences in luminescent intensity of *Armillaria* and observed that only one of nine *Armillaria* spp. (*A. nabsnona*) was consistently luminescent across all replicates and time intervals.

While variation may be masked in the dikaryotic nuclear state (or diploid as is the case for *Armillaria*), such variation is more readily observed in haploid mycelia (SSIs). Here, a comparative analysis of SSIs was undertaken to observe the luminescence intensity of haploid progeny that showed variation in the luminescence phenotype for this trait. In this study, luminescence intensity was consistently higher for SSIs from matings between isolates with a greater luminescence intensity. Furthermore, the frequency of SSIs obtained from matings between bright isolates also produced a higher frequency of isolates with brighter luminescence intensity than either dim \times transformant or intermediate \times intermediate matings. This result is consistent with observations of SSIs of *P. stipticus* that showed a similar variance in luminescence intensity in populations from Japan and North America (Petersen and Bermudes 1992).

The patterns observed in this study were also consistent with increasing temporal luminescence in *Armillaria*. Temporal variation was observed with an increase in luminescence intensity from 14-32 days. Petersen and Bermudes (1992) noted a similar increase in luminescence intensity of *Panellus stipticus* measured from 5-30 days. In contrast, Mihail (2015) observed no consistent increase in luminescence intensity of *Armillaria* over time; however, luminescence intensity of haploid and diploid cultures was measured between 1-7 days post inoculation. The low luminescence intensity of isolates observed at 14 dpi may be attributed to the small size of radial growth and the low abundance of the mycelia in culture. This suggests that the age of the culture should be considered when comparing luminescence intensity to observations that were obtained in other studies.

The detection of variable luminescence in *Armillaria* raises the question about the function of this physiological mechanism that results in light emission. The existence of weak or non-luminescent mycelia of *P. stipticus* was also reported (Bermudes et al., 1990; Lingle et al., 1992; Petersen & Bermudes 1992) but the change from weak to bright luminescence intensity remains unclear. To address these questions, characterization and identification of the genes encoding this mechanism is necessary. The genes encoding bioluminescence in the bacterium *Vibrio fischeri* were characterized by cloning and sequencing the *lux* operons transformed in *Escherichia coli* (Engebrecht et al., 1983). This approach is more challenging for sexually-reproducing bioluminescent eukaryotes with larger and more complex genomes and for these reasons, only a few studies have been attempted. For example, RNA-seq was used to identify three potential proteins

involved in the bioluminescence of the glowworm, *Arachnocampa luminosa* (Sharpe et al., 2015) and analysis of cDNA was used to determine the nucleotide sequence of the luciferase gene in the eastern firefly, *Photinus pyralis* (de Wet et al., 1987). The genes encoding bioluminescence for luminescent agarics have yet to be characterized and the few studies performed to date have focused on identifying the chemical structure of fungal luciferin and oxyluciferin (Purtov et al., 2015; Kaskova et al., 2017).

The effect of the parental phenotype on luminescence intensity and the variance observed here suggests that this is a complex trait involving the interactions of several genes. The study by Lingle et al. (1992) made a similar conclusion based on the variation in luminescence intensity of SSIs from matings of isolates *P. stipticus*. Dikaryons resulting from compatible pairings between bioluminescent and non-bioluminescent SSIs were always bioluminescent and yet, compatible matings between two non-bioluminescent SSIs also resulted in luminescent mycelia (Lingle et al., 1992). Here, the frequency of low, intermediate and bright luminescent phenotypes of SSIs observed in SSIs of *A. mellea* suggests that this trait has a continuous distribution. However, characterizing heritability and the genes that contribute to, and regulate this physiological mechanism, should be examined in future research to understand bioluminescence in *Armillaria*.

The results presented here suggest that the expression of bioluminescence in *A. mellea* is heritable, exhibits a continuous range of luminescence intensity and is controlled by multiple genes. Variation in mean luminescence intensity and frequency of bright, intermediate and dim SSIs supports the findings of previous analyses of

bioluminescence agarics (Bermudes et al., 1990; Lingle et al., 1992; Petersen & Bermudes 1992; Weitz et al., 2001; Mihail and Bruhn 2007; Mihail 2015). Future research examining heritability and environmental regulation of bioluminescence is needed.

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