EXAMINING THE GENETICS OF MUSHROOM DEVELOPMENT IN THE
CULTIVATED EDIBLE MUSHROOM *FLAMMULINA VELUTIPES*

A Chapter Style Thesis Submitted in Partial Fulfillment of the Requirements for the
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College of Science and Health

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EXAMINING THE GENETICS OF MUSHROOM DEVELOPMENT IN THE
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We recommend acceptance of this thesis in partial fulfillment of the candidate's requirements for the degree of Biology – Master of Science.

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ABSTRACT

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*Flammulina velutipes* is a cultivated edible mushroom with two distinct growth forms: highly pigmented and umbrella-shaped in the wild, but pale and thread-like under cultivation conditions. Recent studies using *F. velutipes* to study gene expression changes during mushroom development have largely ignored tissue-level differences and the normal (wild) growth form. The present study extracted and sequenced *F. velutipes* mRNA from four tissues (mycelium, stipe, pileus, and gills) at four growth stages (primordia, young mushrooms, mature cultivated mushrooms, and mature normal mushrooms) to assemble a transcriptome of 20,909 transcripts from 14,416 potential genes. Analyses identified 2,183 differentially expressed genes (q < 0.05), 1,456 of which matched named proteins in the UniProtKB Agaricales database. Tissue had a much larger impact on gene expression than growth stage did and analysis revealed a stipe-specific hydrophobin (UniProtKB accession G8A517), four stipe-specific cytochrome p450 genes, and one cytochrome p450 specific to the pileus and gills. Genes previously known to be involved in the fruiting process (including *fds*, *fvd16*, MAPK, and WD40 repeat-containing genes) tended to be most highly expressed in actively growing tissues, suggesting that regulating cell growth is a key mechanism during mushroom formation. These results augment existing knowledge of developmental genetics of mushroom-forming fungi.
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CHAPTER I

INTRODUCTION

Mushroom development is a field within mycology that seeks to understand how mushrooms take shape and grow. Mycologists have studied mushroom growth using many means, from simply measuring mushrooms (ex: Gruen, 1969), to using light microscopy (ex: Hammad et al., 1993), to employing advanced molecular techniques (ex: Wu et al., 2019). Yet many basic questions remain unanswered, including how mushroom tissues are defined and how mushroom growth is coordinated and regulated. The present study seeks to answer some of these questions by using RNA-seq to examine gene expression in the mushroom *Flammulina velutipes*.

Gene expression refers to all the genes in a cell that are actively producing mRNA. There are three steps to generate a functional protein: transcription, translation, and post-translational modification. In transcription, the DNA code for a gene is copied onto a molecule called messenger RNA (mRNA). In eukaryotic cells, mRNA is processed and then exits the nucleus and travels to the ribosome, where the next stage occurs. In translation, the ribosome reads the mRNA code and assembles amino acids into a polypeptide chain based on that code. The amino acid chain is then folded and, in many cases, modified via covalent bonding to chemical side chain groups to produce the final protein. Studying gene expression consists of identifying which genes are actively producing mRNA and at what levels, which can help explain a cell’s behavior.
One way to identify expressed genes and quantify gene expression levels is by extracting and sequencing all the mRNA in a tissue sample, a technique known as RNA sequencing (RNA-seq). RNA-seq works by extracting mRNA, preparing a complementary DNA (cDNA) copy, amplifying the copies through PCR, and sequencing the resulting amplicons. This process can be used to identify the genes that produced the mRNA and also quantify how many molecules of cDNA from each gene is present. By comparing amounts of cDNA for each gene, it is possible to calculate relative gene expression levels.

In the present study, RNA-seq is used to examine gene expression changes during mushroom development. The study of mushroom development brings together two very different fields: mycology and developmental biology. The context for this research is necessarily broad and includes background information on fungal biology, mushroom development, developmental biology, characteristics of *Flammulina velutipes* itself, and a review of previous research on gene expression in *F. velutipes*.

**Fungal Biology**

The kingdom Fungi is one of the major kingdoms containing multicellular eukaryotic organisms. Fungi belong to the major clade Opisthokonta, which also includes the kingdom Animalia. Organisms in the kingdom Fungi share several features that distinguish them from other forms of life. Fungi are heterotrophs that use exoenzymes to digest their substrate externally. Simple organic compounds are then transported into the cells and converted to energy. Fungi are non-motile, so they must reach new areas by growing. Fungi grow as either yeast (spherical single cells) or as hyphae (cylindrical chains of cells). Hyphae can branch and fuse with each other to form a network called a
mycelium. Fungi reproduce and disperse using spores, which may be produced through mitosis or meiosis. In fungi, cell division is accomplished without complete dissolution of the nuclear envelope (closed mitosis). Fungal nuclei are small and have compact genomes with little repetitive DNA. The cell walls of fungi are primarily composed of chitin (β-1,4 N-acetylglucosamine chains). Chitin distinguishes fungi from superficially similar organisms such as Oomycota, slime molds, and plants, which instead use cellulose in their cell walls or lack cell walls. Fungal cells are also unique in having the combination of: ergosterol in their cell membranes, a unique lysine synthesis pathway, and glycogen as an energy storage compound.

**Life Cycle**

The typical fungal life cycle is relatively simple. First, a haploid spore germinates and produces hyphae (cylindrical chains of fungal cells), which grow to become a haploid mycelium (network of hyphae). This mycelium is typically capable of producing haploid asexual spores. When this mycelium contacts another compatible mycelium, the two mycelia fuse together. Whether the individuals can fuse is determined by factors encoded by genes at mating type loci. There are a few possible variations on the fungal life cycle once compatible mycelia have fused. In fungi with a simpler life cycle, such as the phyla Chytridiomycota and Zygomycota (and most yeasts, although they form individual cells instead of hyphae), only the nuclei at the point of fusion interact. These nuclei fuse to produce a diploid nucleus and then immediately undergo meiosis. Recombinant haploid spores are formed and then released. In fungi with more complex life cycles, like the phyla Ascomycota and Basidiomycota, the point of fusion gives rise to a new mycelium containing nuclei from both parental mycelia. This dikaryotic mycelium is long-lived and
can produce dikaryotic asexual spores, but eventually some of the hyphal compartments develop into sporocarps, go through meiosis, and produce recombinant haploid spores. These spores are released and the life cycle begins again (Figure 1).

**Hyphae**

Hyphae are the most important structures that fungi form, since they branch and fuse with one another to form the mycelial network. The network structure of the mycelium allows for efficient distribution of resources and enables fungi to penetrate into their substrate. Using a combination of exoenzymes, apical growth, and turgor pressure, hyphae can grow into inhospitable materials such as wood, soil, or even rocks. Other common decay organisms such as bacteria cannot grow into their substrates because they lack the mechanical support and turgor pressure that a mycelium provides.

The simplest hyphae are cylindrical structures that contain many nuclei but are

![Figure 1. Generalized life cycle of species in the Ascomycota and Basidiomycota. Spores are represented as ovals and hyphae are represented as lines. Each part of the life cycle is labeled by its nuclear state: haploid (n), dikaryotic (n+n), or diploid (2n).](image-url)
not divided into separate cells (syncytial). More complex hyphae develop walls called septa that divide the hypha into separate compartments (for convenience, one hyphal compartment can be thought of as one fungal cell). Simple septa are flat doughnut-shaped extensions of the cell walls. The pore in the center of the septum is large enough to allow organelles including nuclei to pass unimpeded from one hyphal compartment to the next. The main role of the septum is to limit the amount of cytoplasm lost when an injury occurs. If the hypha is broken the septum can be easily plugged to limit the amount of cytoplasm lost. For example, in ascomycetes an organelle called the Woronin body moves into and plugs the septal pore when an adjacent hypha ruptures. The sealed septum separates the injured hyphal compartment from intact hyphal compartments and prevents cytoplasm from flowing to the injured area.

The most complex hyphae are found in the phylum Basidiomycota and are partitioned by structures called dolipore septa. In addition to a doughnut-shaped wall, dolipore septa also have a structure called a parenthosome: a hemispherical membrane that fits around the septum hole. Parenthosomes have numerous holes and most organelles can pass freely through them; however, the parenthosome prevents nuclei from traveling between hyphal compartments. In fungi with dolipore septa, each hyphal compartment is dikaryotic, containing two haploid nuclei: one from each parent.

Because nuclei cannot move between hyphal compartments, fungi with dolipore septa typically form structures called clamp connections during mitosis. In order to maintain the dikaryon, both nuclei have to divide at the same time, which requires two separate mitotic planes. The first plane is set up along the short axis of the hypha. During mitosis, a new septum forms along the first plane and separates the two daughter nuclei.
To provide space for the second mitotic plane, a projection forms on the surface of the hypha next to the first mitotic plane. The second mitotic plane is set up inside the projection and parallel to the hyphal wall. During mitosis, a new septum also forms along the second plane and separates the two daughter nuclei. At this point, one hyphal compartment is dikaryotic, one hyphal compartment is monokaryotic, and the remaining nucleus is trapped in a small hyphal projection. To re-form the dikaryon, the hyphal projection grows into a half-doughnut shape centered over the septum created along the first mitotic plane. The projection then fuses with the hyphal compartment on the other side of the first septum and releases the nucleus. This last step ensures both hyphal compartments are dikaryotic. As a consequence of this process, hyphae with dolipore septa are left with half-doughnut-shaped bumps called clamp connections.

**Mushrooms**

The most complex and familiar structures formed by fungi are mushrooms, which can also be called fruitbodies or sporocarps. Mushrooms are macroscopic fungal organs designed for spore production, release, and dispersal. Although fruitbodies are the most noticeable fungal structures, they are only temporary organs. The bulk of the fungus instead consists of assimilative mycelium, which continues growing through the substrate even after the mushrooms die back. Almost all mushrooms are produced by fungi in either the phylum Basidiomycota or the phylum Ascomycota. Basidiomycotan mushrooms are the most familiar mushrooms and include *Amanita muscaria* (the basis for the vast majority of mushroom art) and *Agaricus bisporus* (the most widely cultivated commercial mushroom in the United States; Royse, 2014). Ascomycotan mushrooms include morels and truffles, some of the most highly sought-after edible mushrooms.
Mushrooms take on a wide variety of forms; I consider there to be 22 distinct morphological groups and there are still some mushrooms that don't fit well into any of those groups.

The most familiar and largest morphological group of mushrooms is that of the agarics, or gilled mushrooms. All agarics belong to the division Basidiomycota, although the gilled morphology has evolved multiple times within the Basidiomycota. Agaric mushrooms have three main structures: the stipe, the pileus, and the gills. The stipe is the stalk that holds the gills and pileus up above the mushroom's substrate. Air does not move much near the ground or other surfaces, so the stipe allows the mushroom to escape the layer of stagnant air and access air currents for more efficient spore dispersal. The pileus is the upper surface or cap of the mushroom. Its main role is supportive: the pileus provides a structure from which the gills hang. The gills, also called lamellae, are plates of tissue that form perpendicular to the ground and radiate out from the stipe. Gills are so named because they somewhat resemble the gills of a fish. The gills bear the mushroom's hymenium – the tissue where meiosis occurs and sexual spores are produced. In the Basidiomycota, the end product of meiosis is generally four haploid spores (called basidiospores) that form on the outside of the meiotic cell. When the basidiospores are mature, they drop from the gill face and fall between the gills into air currents to be dispersed. Because the spores drop, the gills must be perpendicular to the ground and the stipe must be tall enough to access air currents.

**Mushroom Development**

The field of mushroom development seeks to understand the events behind the mushroom fruiting process. How does the fungus start with a diffuse network of cells and
then build a compact organ with distinct tissues? Fruitbody formation is one of the most complicated processes in the fungal kingdom, but has received less attention than most other areas of mycology.

**Why Study Mushroom Development?**

There are many good reasons to study mushroom development. One of the major reasons is to learn more about how mushrooms evolved. Fungal fruitbodies are fleshy and therefore rarely fossilize. There are a few mushroom fossils trapped in amber, but these already have gills and look remarkably like modern mushrooms (Poinar & Buckley, 2007). As a result, other methods must be used to understand how fungi evolved specialized structures for spore release. Studying how development-related genes evolved can help fill in the gaps in the fossil record, thereby improving fungal phylogenies, assisting fungal taxonomy, and answering longstanding questions about how and when mushrooms evolved.

Studying development will also improve our knowledge of mushroom cell biology. A lot of work has been done on cell biology in fungi, but most focuses on hyphae and yeasts (e.g. Harris & Momany, 2004; Novick et al., 1981; Roche et al., 2014). Unlike yeasts and hyphae in the assimilative mycelium, mushroom cells are packed together into tissues and must tightly coordinate their actions. The proteins, genes, cellular structures, and communication methods that enable tight coordination in complex multicellular structures have not received much study and are still largely unknown.

There is also an economic incentive to study mushroom development; mushroom cultivation is a large global industry which, after all, relies completely on proper fruitbody development. Development-related research that has already been done for
commercial reasons includes identifying sporeless mutations (fewer spores means fewer respiratory problems for mushroom pickers; e.g. Ravishankar et al., 2006), characterizing mycoviruses that alter development (such as La France Disease in *Agaricus bisporus*; e.g. Wach et al., 1987), and using genetic engineering to prevent postharvest browning in *A. bisporus* (Waltz, 2016). Further research into mushroom development could provide other advantages, such as finding ways to more precisely trigger fruiting or grow larger mushrooms.

Lastly, mushroom hunters have always been interested in mushroom development, which is not surprising. As any experienced mushroom hunter knows, mushrooms often don’t look like they’re “supposed” to: chanterelles can form rosecombs (gill tissue that grows on top of the cap), weather can warp mushrooms, fruitbodies can grow around sticks, etc. A common question among mushroom hunters is, “Why does that mushroom look abnormal?” – a simple question, but one that cannot be answered by current knowledge.

Even though there are many reasons to study mushroom development, this branch of mycology has received little attention. The process of mushroom development is still poorly understood, especially from a molecular standpoint. Much work still needs to be done before knowledge of mushroom development can be put to practical use to answer even the most basic questions posed above.

**The Basic Model of Mushroom Development**

A basic model of mushroom development underlies the current understanding of mushroom formation. Before beginning fruitbody formation, the fungus must sense that it has enough stored energy and that the external conditions (e.g., humidity, temperature,
and sunlight) are favorable (Moore, 1998). Once conditions are met, the fungus can start producing mushrooms:

1. A ball of cells forms on the mycelium. This ball is known as the “fruitbody initial” and is little more than a loose clump of hyphae (Moore, 1998).

2. The hyphae in the fruitbody initial grow and become denser, forming a structure called the primordium (Moore, 1998). The primordium is still a ball of hyphae, but tissues begin to appear inside the primordium. By the time the primordium is fully formed, the gills, stipe, and pileus are all present inside the structure.

3. The mushroom expands. Because specific tissues are already formed in the primordium, the mushroom can easily get bigger by simply inflating its cells. Mushroom expansion is similar to inflating a balloon: the hyphae swell with water, thereby expanding the whole fruitbody (Moore, 1998). Different morphologies can be created by altering the arrangement of cells in the primordium and also by changing the patterns of tissue expansion.

Evidence of this three-step process of fruitbody formation is readily found in nature. Mushroom collectors and cultivators are very familiar with the fruitbody initial: these tiny bumps appear on the surface of the substrate (whether it’s a log, bundle of hay, or a petri dish) and mark out the places where mushrooms will grow. Primordia are particularly easy to see in *Amanita* species, since they are typically around the size of a golf ball. When sliced in half from top to bottom, the immature pileus, gills, and stipe are easily visible inside the *Amanita* primordia (Figure 2). *Amanita* species also provide a
good example of growth by cell expansion. In many species, the universal veil (a membrane covering the outside of the primordium) will break apart to leave warts on the pileus (Figure 2). The warts are initially close together and evenly spaced. As the mushroom expands, the warts become further apart but remain evenly spaced. This phenomenon demonstrates that no tissue is being added to the cap. If tissue had been added to the middle of the pileus, the warts would have been pushed to the edges and ended up in a ring shape. If tissue had been added to the edges of the pileus, the warts would have remained close together at the center of the pileus.

However, there are also many examples in nature that cannot be explained by this model. The growth by cell inflation model would predict that two mushrooms growing next to each other would push each other out of the way and remain separate. Many polypores (mushrooms with pores underneath, excluding the bolete lineage) will instead fuse with nearby mushrooms, a behavior that is not predicted by the basic model.

Figure 2. Stages of sporocarp development in *Amanita* mushrooms. A. The base (b), stipe (s), gill (g), and pileus (p) tissues are visible in a cross-section of an *A. bisporigera* primordium. B. The universal veil (u) of *A. muscaria* var. *guessowii* breaks up into warts (w) that at first are close together but become evenly spaced out over the pileus as the cap expands. Photos by Thomas Roehl.
Determinate Growth, Indeterminate Growth, and Haptomorphosis

To account for developmental behaviors not predicted by the basic model, mycologists recognize two major types of development in mushrooms: determinate growth and indeterminate growth. Determinate growth describes a situation where a structure grows to a specific size and shape determined by its genes (Nagy et al., 2018). Indeterminate growth, on the other hand, describes structures that do not have a predetermined size and shape: the structure will continue to grow until it reaches an external constraint (Nagy et al., 2018). Both growth forms are easily recognized in the field by looking at the pattern of mushroom growth.

Determinate growth can be recognized when the mushroom pushes aside debris and other nearby mushrooms. Gilled mushrooms are good examples of determinate growth: *Amanita* species push dirt out of the way when they burst through the soil, and *Agaricus* species can even push through concrete; *Pleurotus, Flammulina,* and *Armillaria* species often grow in dense clusters but their caps are always separate from one another. Pushing aside nearby objects is a consequence of growth by cell inflation, which is the backbone of the basic developmental model. The model was created after studying gilled mushrooms (Moore, 1998), which use determinate growth. Consequently, the model works well to predict the behavior of determinate species.

Indeterminate growth can be recognized when mushrooms incorporate debris into their fruiting bodies or fuse with the mushrooms around them. Many polypores exhibit this type of growth: *Trametes versicolor* and *Trichaptum biforme* often fuse with one another to completely cover the surface of a log; *Phaeolus schweinitzii* and *Inonotus dryadeus,* since they grow close to the ground, usually have leaves, sticks, and blades of
grass sticking right through the mushrooms. The tendency of indeterminate mushrooms to grow around or engulf debris is known as haptomorphosis (Reijnders, 1991). Growing around obstacles and fusing with nearby mushrooms are both actions that indicate new cells are forming on the outside of the mushrooms. Rather than simply inflating existing tissues, indeterminate growth fungi add new tissue to the mushroom as they grow. Adding new tissue is not accounted for in the basic developmental model, which means the model cannot be applied to indeterminate mushrooms.

Even the determinate/indeterminate growth categorization system has its limitations; there are many examples of mushrooms that don’t quite fit into either group. For example, you often find boletes that push aside debris but have fused pilei, demonstrating some features of each type of growth. Furthermore, some mushrooms seem to use different types of growth in different parts of their fruitbodies. For example, *Gyromitra brunnea* usually has a lot of dirt embedded in its stipe, but the cap pushes nearby objects out of the way. Unusual growth patterns like those have never been studied from a developmental standpoint and therefore cannot be explained by current theoretical frameworks.

**Developmental Mycology**

To overcome the limitations of current fruitbody development models, mycologists can employ concepts from the field of developmental biology. Developmental biology seeks to understand how organisms start out as one cell or a small collection of identical cells and change to become large complex organisms with distinct organs and tissues. Biologists researching development typically focus on the processes or mechanisms underlying developmental events. The processes and mechanisms are
repeated across widely diverged species and even kingdoms. As a result, these concepts can be broadly applied to all forms of complex multicellular life.

**Complex Multicellularity in Fungi**

In fungi, development has been studied at two levels: the development of individual hyphae and the development of fruiting bodies (e.g. Chiu & Moore, 1990a; Harris & Momany, 2004). However, the study of developmental biology focuses on the formation of complex multicellular structures. Hyphae are not complex structures and hyphal development therefore does not fall under the category of developmental biology, even though understanding how hyphae organize themselves and coordinate their growth with the rest of the mycelium is fundamental to understanding fungi. The sporocarp, on the other hand, is a complex multicellular structure and concepts from developmental biology can be directly applied to mushroom development.

Both mycelium and mushrooms are considered to be multicellular structures. That mushrooms are multicellular is easily observed simply by looking at them: they are large structures composed of many physically and functionally distinct hyphae (Corner, 1932b). The mycelium, however, is a little more difficult to characterize. In “lower” fungi, there are no divisions in the mycelium to separate distinct cells and each coenocytic hypha contains many nuclei (Nagy et al., 2020). Even in lineages (Ascomycota and Basidiomycota) that routinely divide their hyphae into separate compartments, contents of one compartment can easily flow into an adjacent compartment. Because hyphae are functionally coenocytic, the mycelium could be considered unicellular. However, the nuclei within each hypha are arranged and proliferate in a similar pattern to cells in multicellular organisms; therefore, mycologists
consider the mycelium to be multicellular (Nagy et al., 2020).

The question then becomes whether fungal structures are complex. There are many different definitions of “complex,” some of which include the mycelium (e.g. Gonçalves et al., 2019) and others that exclude the mycelium (e.g. Knoll, 2011). Nagy et al. (2020) provide a definition that is particularly useful for fungi: complex multicellular structures are compact three-dimensional structures in which some cells are not exposed to the external environment. If the cells all contact the environment, all the cells must be able to perform the same functions, such as extracting nutrients, defense, and transport. However, when cells are protected from the external environment, they no longer need to perform some of those functions and can become specialized to complete specific tasks.

Cell specialization is a hallmark of complex multicellular life: cells performing different functions work together to form tissues and organs that carry out specific and necessary functions to benefit the organism as a whole (Nagy et al., 2020). How cells specialize and form tissues is a major focus of developmental biology; a focus on compact three-dimensional fungal structures with interior cells is therefore a fitting definition for the goals of developmental mycology.

The largest structure formed by a fungus is the assimilative mycelium, but is mycelium a complex structure? The mycelium is certainly three-dimensional: it can grow in all directions depending upon the shape of its substrate and location of external nutrients. However, the mycelium is not compact, nor does it have interior cells. The main role of the mycelium is to extract nutrients from the external environment. To accomplish this task, hyphae are arranged in a diffuse network and all hyphae contact the external environment. This ensures hyphae are arranged for maximally efficient resource
extraction from the substrate (Nagy et al., 2020). Because the hyphae in the mycelium are not compact and all contact the environment, the mycelium is merely a simple multicellular structure (Nagy et al., 2018, 2020).

Although the main body of the fungus is considered a simple form of multicellularity, there are specialized fungal structures that fit the definition of complex multicellularity. These structures include: mushrooms, sclerotia, rhizomorphs, and mycorrhizae (Nagy et al., 2020). Of these, the most extensively studied structures are mushrooms. Mushrooms form a wide variety of three-dimensional shapes, are composed of mycelium closely bundled together, and usually have morphologically different external and internal hyphae. Consequently, mushrooms are true complex multicellular structures (Nagy et al., 2020). In the context of developmental biology, a mushroom or fruiting body can be considered a spore-producing structure in which many supportive cells do not contact the external environment. Mushrooms come in many forms and that definition is unspecific by necessity. As defined, this term includes the “mushrooms” widely sold in grocery stores, barely visible structures such as perithecia, and microscopic synnemata (which have relatively few specialized cells, but there are supporting cells completely enclosed in the structure). Fruiting bodies by this definition are found mostly within the Ascomycota and Basidiomycota, but some are produced by zygomycetes (Knoll, 2011; Nagy et al., 2020; Yamamoto et al., 2015). Most mushroom developmental studies, including the current study, have focused on mushrooms from the Basidiomycota (Nagy et al., 2020).


**Applying Developmental Biology to Mycology**

The development of complex three-dimensional fungal structures has been studied most intensively in the context of mushroom formation. However, even fruitbody formation is rarely studied from a purely developmental biology perspective. This is partially because developmental biology research primarily uses animal models. Animal research is highly emphasized because it can be easily applied to humans and used to understand human developmental problems (for example, birth defects and cancer). Despite the focus on animal research, developmental biology still provides a useful framework for understanding fungal development. All complex multicellular organisms must solve similar problems to create their final shapes and consequently use similar strategies to do so. Because developmental biologists focus on those strategies, major concepts from developmental biology are applicable to any form of complex life.

The major strategies found in all multicellular life are divided into five mechanisms and four processes. Mechanisms are the small-scale events that are used to carry out the processes and consist of protein expression, cell-to-cell communication, cell growth and division, programmed cell death (apoptosis), and cell migration (adapted from Wolpert et al., 2011a). Processes are overarching events that have to occur for development to be successful: pattern formation, morphogenesis, cell differentiation, and growth (Wolpert et al., 2011a). Mycologists rarely discuss their research from the standpoint of these developmental mechanisms and processes, but most previous mushroom research can be reframed into this developmental biology context.
Developmental Mechanisms

The five basic developmental mechanisms are tools used over and over again in different contexts to carry out all developmental tasks. Studying the mechanisms takes a small-scale look at development: what is happening in a single tissue or a single cell or at a specific point in time to move development forward?

Protein Expression. Ultimately, what a cell does is determined by the proteins it produces. By changing the proteins present, the cell can alter its function or behavior or become specialized to performing a specific task. Fungi are known to change the proteins expressed in different tissues as development progresses (De Groot et al., 1996; Lugones et al., 1999; Sakamoto et al., 2007). Differences in protein expression are presumably responsible for most of the physical differences in hyphae seen between tissues and even within tissues.

Some of the most-studied proteins in mushrooms are hydrophobins. These are short proteins with a hydrophilic half and a hydrophobic half (Lugones et al., 1998; Wösten et al., 1994). Hydrophobins are particularly important when hyphae are growing in the air. They self-assemble into a layer of rods that coats the hyphae. The hydrophobin layer is oriented with the hydrophilic side against the cell to help keep water in and the hydrophobic side facing out to form a barrier against the external environment.

Because hydrophobins are important for aerial hyphae, these proteins are often specifically expressed in fruitbodies. *Agaricus bisporus*, *Schizophyllum commune*, and *Flammulina velutipes* all have specific hydrophobins that are produced only in mushrooms (Ando et al., 2001; De Groot et al., 1996; Lugones et al., 1996, 1998; Wösten et al., 1994). These hydrophobins presumably help the mushroom maintain its structure –
even gaps inside the mushrooms are coated with hydrophobins (Lugones et al., 1999). Additionally, *F. velutipes* produces different hydrophobins for different tissues, including one (PSH) that is only produced by cells in the pileus (Sakamoto et al., 2007).

**Cell-to-Cell Communication.** Cells are constantly communicating with their neighbors using molecular signals. There are many types of cell-to-cell communication, and all can be used to perform developmental tasks. Some molecular signals establish gradients by diffusion, which can be used to trigger differential gene expression (Johnston & Nüsslein-Volhard, 1992; Rivera-Pomar & Jäckle, 1996). Other secreted signals coordinate development throughout the entire organism (Swain & Lovell-Badge, 1999). Signaling through direct contact between two cells can even be used to convey positional information (Marthiens et al., 2002; Suter & Forscher, 2000). All these methods of cell-to-cell communication ultimately change cell behavior to achieve specific developmental outcomes.

Because of the structure of fungal cells, there are unique challenges for cell-to-cell communication in fungi. Fungal cells are arranged in coenocytic hyphae; cytoplasm and any signaling molecules contained therein can therefore pass freely from one cell to the next. Cell-to-cell communication along a single hypha is consequently easy; the signal can quickly travel through the septum to the next cell without requiring membrane transport. The same hyphal structure makes it difficult to communicate between cells in adjacent hyphae: the signal must exit one cell, cross the cell wall, diffuse through the space between hyphae, cross the other cell wall, and then enter the other cell. Although hyphae do link with adjacent hyphae through anastomoses (lateral points of fusion with nearby hyphae), these connections are too infrequent to be responsible for all
communication between hyphae (Rosin et al., 1985; Williams et al., 1985).
Consequently, fungal cell-to-cell communication exists in an asymmetrical context:
sending a signal along one hypha can be done quickly, but sending a signal between two
adjacent hyphae is slow.

Cell-to-cell communication in fungi has primarily been studied in the mycelium.
We therefore know a lot about how fungal cells communicate in the assimilative
mycelium. Communication between hyphae in the mycelium ensures that hyphae are
evenly spread throughout the substrate to ensure the most efficient use of resources and
controls hyphal branching and fusion (Bartnicki-Garcia, 2002; Fischer & Glass, 2019;
Glass et al., 2000).

In mushrooms, however, hyphae are compact and must communicate differently.
These mushroom-specific cell-to-cell communication processes have not received as
much attention and are therefore poorly known. Most efforts have centered on identifying
mushroom growth hormones (Moore, 1998). Hormones are compounds that act at a
distance and cause specific cellular responses. Testosterone is an example of a hormone
found in mammals that is produced in the testes once those cells differentiate but triggers
the development of male characteristics in the rest of the embryo (Swain & Lovell-
Badge, 1999). There is some good evidence that fungi also use hormones to coordinate
development, but so far, no mushroom hormones have been definitively identified
(Moore, 1998).

Stipe growth seems to be coordinated by hormones, at least in some species.
Cutting off the cap in A. bisporus and F. velutipes will cause the stipe to stop growing
(Gruen, 1963, 1969). Presumably, stipe growth ceases because the stipe grows in
response to a signal from the cap. This signal is acting at a distance and therefore would seem to fit the definition of a hormone. However, attempts to find chemicals that impact stipe growth have produced inconclusive results (Moore, 1998).

Other good evidence for hormones comes from a study of *A. bisporus* and *S. commune*. An extract prepared from *A. bisporus* mushrooms can be applied to *S. commune* mycelium to induce mushroom formation (Rusmin & Leonard, 1978). In this case, the *A. bisporus* mushrooms presumably contain some kind of signal hormone that promotes mushroom growth. *S. commune* apparently uses the same signal, which is why mushrooms start growing after the liquid is applied. Once again, attempts to identify the hormone was did not produce useful results. As it stands, nobody has proven that mushrooms use hormones to communicate, even though it appears as if they do (Moore, 1998).

**Cell Growth and Division.** Tissues can grow either by making the cells larger (cell enlargement) or by making more cells (cell division). Mushroom tissues are generally enlarged using either cell growth or cell division. Some mushrooms, such as most gilled species, get bigger by inflating all their cells (cell growth), which causes the mushroom to expand like a balloon. This pattern is typical of mushrooms that use determinate growth. Other mushrooms, including most polypores, enlarge by adding new layers of cells to the outside of their fruitbodies (cell division). To produce the next layer, new hyphal tips form and extend away from the fruitbody. This pattern is typical of indeterminate mushrooms.

In the typical model of mushroom development (used by determinate mushrooms), fruiting bodies get bigger through the mechanism of cell growth. As the
individual cells inflate, the mushroom itself gets larger. Hammad et al. (1993) demonstrated this process of growth-by-cell-expansion using research termed “quantitative hyphal analysis,” which measures hyphal diameters inside mushrooms. They measured cells in *Coprinopsis cinerea* as it grew found that cells do not always grow uniformly. In that species, the cells in the stipe primordium are tightly packed and uniform in size. However, in the mature mushroom, the hyphae in the second to innermost ring of the stipe are much larger while the surrounding cells are still roughly the original size. Because the cells in the very center and around the outside do not change size, they are drawn to the edges of the inflating ring. The end result is a stipe that is bigger but has a hole in the middle. By using cell growth in a specific area of the stipe, the mushroom both makes itself larger and changes its shape (Moore, 1998).

The typical developmental model does not apply to all mushrooms and as a result the idea that cell growth drives fruitbody growth does not work in every case. Consequently, the growth-by-cell-expansion model should be applied only to gilled mushrooms.

**Programmed Cell Death.** Programmed cell death is an orderly process where the dying cell is disassembled into small compartments, which are then consumed and recycled by nearby cells (Jacobson et al., 1997). Cell death is a common developmental mechanism in animals. For example, programmed cell death is used in chickens, mice, and humans to remove the webbing between a fetus’s fingers (Zuzarte-Luis & Hurle, 2005). The timing and location of this type of cell death is tightly controlled by internal and external signals, allowing programmed cell death to be used in a highly specific manner (Jacobson et al., 1997). Programmed cell death has not been researched
thoroughly in mushrooms, so there are only a couple examples of the phenomenon: gill deliquescence in *Coprinopsis cinerea* and gill formation in *Agaricus bisporus*.

Early on in the study of mushroom development, programmed cell death was recognized as an important feature in the development of *C. cinerea*. In that species, the gills liquefy beginning at the outermost edge and continuing toward the base of the gill (Buller, 1924). This is a specific pattern that happens every time the mushroom matures, demonstrating that the cell death is indeed programmed. However, compared to most examples of programmed cell death, inky cap gill deliquescence is unusually messy. The cells of *C. cinerea* use enzymes to break down the cell walls around their dying hyphae, letting the cell contents spill out (Iten & Matile, 1970). Crucially, the messy nature of this kind of programmed cell death is advantageous to the mushroom: the extra unorganized results from the tissue autodigestion, which is believed to benefit spore dispersal in this and other inky caps. By liquefying the outer edges, *C. cinerea* can remove parts of the mushroom that have already released spores, which decreases the obstacles between newly maturing spores and clear air currents. Although inky cap deliquescence does not fit the normal pattern of programmed cell death, it is still an example of the phenomenon because it is a specific process that occurs at one defined developmental stage and serves a useful purpose in the organism.

Programmed cell death has also been researched in *A. bisporus*, but not to the same extent. In that species, cell death is used to create the gaps between the gills early on in mushroom development (Umar & Van Griensven, 1997). Programmed cell death here is a much more orderly process and does not leave behind extraneous fluid. This procedure aligns much more closely to the traditional concept of programmed cell death,
where cells die in an orderly fashion to create morphological features. Similarly, Sakamoto (2010) suggested that orderly cell death is responsible for creating the fracture between the pileus margin and stipe during *F. velutipes* development. It is likely that many other examples of this type of programmed cell death can be found in mushroom development but simply have not been researched.

**Cell Migration.** Cell migration is a mechanism that involves groups of cells moving from one place to another. This mechanism can be very important for development, such as when a population of cells originates in one area but has to get to a different area to carry out its function. For example, during human brain development, neurons crawl up a scaffold of glial cells in the brain to form specific layers of tissues (Budday et al., 2015). The mechanism of cell migration therefore plays a vital role in proper brain formation. Of course, plant and fungal cells do not move – their rigid cell walls make that impossible. Consequently, cell migration is not a mechanism used in plant or fungal development.

**Developmental Processes**

The four major developmental processes are large-scale procedures that bring together multiple mechanisms to define what the organism will look like, how big it will be, etc. Pattern formation, morphogenesis, cell differentiation, and growth occur in all complex multicellular life forms, making them useful for comparing development in distantly related organisms as well as closely related ones.

**Pattern Formation.** Proper pattern formation is vital to any complex organism and usually happens early on in an organism’s life. For example, even before a fruit fly embryo is fertilized, molecular gradients define the anterior-posterior and dorsal-ventral
axes of the cell (Johnston & Nüsslein-Volhard, 1992). After fertilization, each nucleus responds to the local concentrations of those molecules by activating a specific set of genes. Different concentrations will activate different sets of genes, resulting in nuclei that behave differently in different parts of the embryo. Some of the newly activated genes will establish new concentration gradient patterns, which in turn further alter gene expression in nearby nuclei (Rivera-Pomar & Jäckle, 1996). This sequence of molecular patterns altering gene expression continues and cells become specified to certain developmental pathways, eventually resulting in tissues and organs forming in specific areas of the organism.

In mushrooms, pattern formation occurs in the primordium. This process is what results in the stipe, pileus, and gills visible inside Amanita primordia (Figure 2). By the end of the primordial stage, pattern formation is complete and the tissues simply need to be expanded.

The early steps in pattern formation have mostly been studied in gill tissue. Initially, gill tissue forms following a simple rule: “Where there is space, make gill” (Moore, 1998, p. 368). This is a very basic pattern formation rule and consequently results in convoluted wrinkles.

After the basic pattern is established, that pattern is continued by the work of organizing centers (Moore, 1998). Organizing centers are small areas of cells that control pattern formation in the nearby cells. The organizing centers for the gills are located between adjacent gills near the gill bases (Moore, 1998). The cells in that area direct cells on the right and left sides to become gill tissue. As this happens, the organizing center moves upward into the cap (Rosin & Moore, 1985). Essentially, gill tissue is not added,
but rather space between gills is excavated out of the pileus tissue. When organizing centers get too big (there is too much space between the gills), they split in half, which results in a new gill forming between two established gills (Rosin & Moore, 1985). This process accounts for the existence of short-gills (lamellulae), but does not explain what causes gills to fork. Much more research needs to be done before we understand how these organizing centers form and are regulated.

Finally, the tissue is pulled into straight lines using mechanical stretching forces: either the pileus expands or the cells in the gills themselves inflate. In both cases, the wrinkles are subjected to stretching, which pulls them into neat rows (Chiu & Moore, 1990b, 1990a). The same phenomenon acts when folding a sheet: when the sheet is pulled tight from both sides, parallel lines will appear stretching from one side to the other. Mushrooms use this same mechanical process to arrange the wrinkles into regular linear ridges (Moore, 1998).

A simple experiment demonstrates that gills do not extend from the tip: marking the gill margins with ink. In Volvariella bombycina and Pleurotus pulmonarius, the marks remain at the bottom of the gill even as the gills get bigger (Chiu & Moore, 1990b; Moore, 1998). The tip, therefore, cannot be the area controlling gill formation. This agrees with previous research from C. cinerea that gills are organized from the base of the gills (Moore, 1998). These three species belong to three different families of gilled mushrooms, suggesting that basal gill organization is widespread among agarics.

**Morphogenesis.** Morphogenesis focuses on how a ball of cells changes its shape to form various structures. In mushrooms, morphogenesis is mostly seen after the primordium stage. Consider an Amanita: all the tissues are packed into the egg-shaped
primordium (“button”), but a fully grown Amanita mushroom is umbrella-shaped. At some point in its development, the mushroom’s shape had to change: this is the idea behind the process of morphogenesis.

Cell growth (which facilitates mushroom growth, as described above) also drives morphogenesis. A detailed demonstration of this phenomenon comes from “quantitative hyphal analysis” (measuring hyphae) in *Coprinopsis cinerea*. The pileus of *C. cinerea* starts out egg-shaped. As the spores mature, the cap opens up to become conical and finally the edge flares up to make the cap bell-shaped. By measuring the cells in the cap, researchers demonstrated that this changing morphology is driven by cells in the cap growing at different rates (Moore et al., 1979). The cells at the very top of the pileus actually don’t get bigger at all, while cells in the gills expand greatly. Because the tissue underneath the cap gets bigger while the cap remains the same size, the gill tissue pushes the cap upward, eventually causing it to flare like a bell. In this way, expanding cells in particular places in the mushroom can be used to create a variety of shapes.

Cell growth clearly plays a large role in morphogenesis, but other factors can also be used to create shape. A good example of this is found in marking experiments on *Pleurotus pulmonarius* (Moore, 1998). When the gill edges of that species are inked, the marks stay near the center of the mushroom even as the cap gets bigger. The end result is lamellae where the inner (older) half of the gill is marked while the outer (newer) half of the gill is unmarked. This phenomenon of half-marked gills cannot be explained by simple cell expansion. If cell expansion were solely responsible for growth, the entire lamella edge should have remained marked. Growth in *P. pulmonarius*, therefore, must be driven by cell division (adding new tissue) in addition to cell expansion. *Pleurotus*
*pulmonarius* is fan-shaped, a distinct morphology that is likely a result of using both cell growth and division for morphogenesis. Similar slight differences in the mechanisms used to drive morphogenesis are likely responsible for many of the diverse shapes present in agarics.

**Cell Differentiation.** In complex organisms, not all cells are the same. Humans, for example, have skin cells, brain cells, muscle cells, etc., all of which look different and perform different functions. Cell differentiation is the process through which these differences are established. Cells within mushroom tissues also look different from each other and perform specialized functions. These differences are primarily controlled by protein expression, which is the focus of much recent mushroom development research, as described above. Cellular differences have also been studied from a physical standpoint, primarily using light microscopy.

One of the earliest ways to understand how mushroom cells differ from each other was to describe the physical differences between hyphae. This microscopic examination of hyphae was termed “hyphal analysis.” Although hyphal analysis was most popular in the 1930s, the categories of hyphal morphology are still useful today when identifying polypores and crust fungi (small mushrooms that lie flat against their substrate). These categories are less useful from a developmental standpoint, but do provide one major insight: cells inside tissues can look different. Edred J. H. Corner (1932a, 1932b) classified hyphae into three different types: generative, skeletal, and binding hyphae. Each cell type is visually distinct and they are assumed to have distinct functions. At some point during development, these cells became different from one another to take on specific roles in the mushroom. How those cells underwent differentiation has not been
studied, but warrants further research by developmental mycologists.

Cell differentiation has been tracked in the greatest detail in gill tissue. For both *Agaricus bisporus* and *Coprinopsis cinerea*, mycologists have tracked the origin and fate of each cell in the hymenium. In both species, the surface of the gill is composed of three types of cells: basidia (which produce the spores), cystidia (which are large and stick out of the gill surface to perform an unknown function), and support cells (which provide structure). In *A. bisporus*, the support cells (termed “basidioles” in that species) appear first and make up the entire gill surface early in development (Allen et al., 1992). Later, some of the support cells get larger and differentiate into basidia and cystidia. All three types of cells come from the same original population of cells. In *C. cinerea*, cells similarly form a layer over the gill surface (Rosin et al., 1985); however, all of the cells in that layer develop into basidia and cystidia. Later on, cells (termed “paraphyses” in *C. cinerea*) from the inside of the gill grow into the layer of basidia and inflate to form the support cells. The three types of cells are therefore derived from two separate cell populations. That the support cells form in such different ways is surprising since it indicates that different cell differentiation programs are used to form structures that carry out the same function. Does this mean that gills evolved separately in the two groups, or perhaps that forming support cells late is important for gill deliquescence? Answering questions like these has the potential to inform our understanding of gill evolution in both groups.

**Growth.** The process of growth takes a large-scale look at how the organism becomes bigger. For mushrooms, growth is either a result of cell growth (the mushroom inflates like a balloon) or cell division (new layers of tissue form on the mushroom’s
exterior). Both of those phenomena are discussed above in the “Cell Growth and Division” section. The process of growth was also discussed under “Cell-to-Cell Communication” in the context of mushroom hormones. In addition to those two contexts, fruitbody growth has been studied at the level of individual genes.

The ultimate goal for mycologists researching mushroom growth is to understand what genes control growth and how they interact with each other. So far, several genes have been identified, but how they link together is still unknown. One of the most interesting genes is \textit{eln2}, which encodes a cytochrome P450 in \textit{Coprinopsis cinerea}. When \textit{eln2} is mutated, the stipe of \textit{C. cinerea} will not expand (Muraguchi & Kamada, 2000). Cytochrome P450s have many roles in the cell, which suggests the \textit{eln2} gene is just one step in a long pathway that controls stipe expansion. Having this as a starting point should help determine the other genes that trigger and carry out stipe growth.

Another mutation in \textit{C. cinerea} to the gene \textit{ich1} prevents pileus formation. This gene appears to act in the nucleus, so it could be near the beginning or the end of the pileus formation pathway (Muraguchi & Kamada, 1998). One early gene involved in \textit{C. cinerea} mushroom formation is \textit{pcc1}, which can trigger mushroom production when mutated.

The \textit{pcc1} gene interacts with mating type genes, which promote a specific type of cell-to-cell signaling (Murata et al., 1998). These likely represent the first couple of links in the developmental pathway that leads to mushroom formation, although how it all fits together is not yet understood.

One pathway that is well understood is the spore production pathway, which is underpinned by meiosis. Meiosis itself has been studied extensively, so it is not surprising that spore production is well understood (Moore, 1998). It also helps that many
mutant strains that fail to produce spores have been developed for *C. cinerea* (Kanda et al., 1989) as well as for some cultivated mushrooms. By studying the point at which meiosis fails and the genes responsible for each failure, mycologists have been able to reconstruct the steps controlling spore formation (Moore, 1998). Because spore development focuses on individual cells instead of tissues, it does not tell us much about overall mushroom development.

**The Future of Developmental Mycology**

Clearly, mycologists know pieces of the mushroom development puzzle but very little about how those pieces fit together. Moving the field forward will require concerted efforts to research those links. Much of the recent research has focused on cell differentiation. Using new genetic tools, researchers are starting to understand what makes tissues different from the level of individual genes. The more we learn about gene activity, the more we can start applying genetic tools to other areas such as pattern formation. Once a gene of interest is identified, it can be manipulated in coordination with microscopy to figure out when, where, and how that gene is active. The present study falls at the beginning of this process: it seeks to characterize gene expression differences in *F. velutipes* mushrooms, which will enable future researchers to probe genetic pathways in more detail.

**Flammulina velutipes**

*Flammulina velutipes* (Curtis) Singer is a determinate agaric mushroom (Figure 3) that is used as a model organism for mushroom development. *Flammulina velutipes* belongs to the division Basidiomycota, order Agaricales (which includes most gilled mushrooms), and family Physalacriaceae (Matheny et al., 2006). Physalacriaceae is a
small family that includes some common wood decay agarics such as *Oudemansiella furfuracea* and *Armillaria*, a genus that includes several severe tree pathogens that have a large economic impact on the forestry industry. *Flammulina velutipes* itself is a wood decay fungus that grows as hyphae in rotting hardwood logs across the northern hemisphere (Bessette et al., 1997; Miller & Miller, 2006). The fungus causes a white rot, which means it primarily decays the lignin in the wood, leaving the cellulose intact.

Mushrooms of *F. velutipes* typically appear in the fall or spring, but can also appear during warm weather in the winter and cold weather in the summer.

Fruitbodies of *F. velutipes* are small to medium in size, umbrella-shaped, and typically grow in clusters (Bessette et al., 1997; Miller & Miller, 2006). The pileus grows to 1.5-10 cm in diameter. When young, the pileus is convex, but it flattens out as the mushroom matures. The pileus can be yellow, orange, or (less frequently) brown, and is usually tinged with reddish tones. There is often a darker area over the center of the

Figure 3. Comparison of the two growth forms of *F. velutipes*. Left: the cultivated form of *F. velutipes* features pale colors, a long stipe, a small pileus, and underdeveloped gills. Right: the normal form of *F. velutipes* has a dark velvety stipe, well defined gills, and a large orange slimy pileus. Photos by Thomas Roehl.
pileus. The pileus margin is curled under when young and develops striations in age. When fresh, the pileus is smooth but covered in a slimy coating produced by a layer of gelatinized hyphae beneath the cap surface. Older or dried out mushrooms usually remain slightly sticky. The flesh of the cap is whitish to yellowish, has a soft texture, and lacks any distinct odor or taste. Under the microscope, clamp connections are visible on the hyphae that compose the flesh tissue.

Underneath the pileus, the lamellae are creamy white to yellowish with close or subdistant spacing (Bessette et al., 1997; Miller & Miller, 2006). The gills attach to the central stipe (slightly to broadly) and in age may start to run down the stipe slightly. The margins of the gills may appear slightly hairy. Lamellae release the spores and leave a white spore print. The spores are elliptical, measuring 7-9 µm long and 3-6 µm wide. Under the microscope, the spores are smooth, clear, and do not change color in Melzer’s Reagent (a reagent that tests amyloidity).

The stipe grows 2-8 cm tall and 0.3-1.2 cm wide (Bessette et al., 1997; Miller & Miller, 2006). Near the top, the stipe is yellowish and smooth. The rest of the stipe starts out yellowish, but darkens to dark reddish brown or black and becomes covered with dark velvety hairs as the mushroom ages. Initially, the dark colors and fuzz appear near the base of the stipe, but they progress upward during mushroom maturation. The flesh of the stipe is tough and hard to break.

*Flammulina velutipes* uses a tetrapolar mating system, with mating type genes divided into two loci: mat*A* and mat*B* (van Peer et al., 2011). The mat*A* locus contains three genes that encode transcription factors. The mat*B* locus contains seven genes that are related to pheromone production and pheromone receptors.
Growth Forms

*Flammulina velutipes* can produce two different forms of mushrooms depending on the growing conditions (Figure 3). The form described above is the normal form and is how the mushroom appears in the wild. When cultivated, however, the mushroom takes on a different appearance. The cultivated form of *F. velutipes* features a small pileus (0.2-1.5 cm) that is hemispherical and white (Wang et al., 2015). The gills are underdeveloped and white. The stipe is also white and elongated, growing to a length of 10 cm long or longer. The whole mushroom is smooth and has a soft but firm texture.

To obtain the cultivated form, mushroom growers keep the mushrooms in the dark and expose them to high levels of carbon dioxide (Stamets, 1993). Darkness is particularly important because the pileus will expand when exposed to light (Sakamoto et al., 2002). These conditions can be easily created by putting a paper or plastic collar around the jar in which the mushrooms are growing (Stamets, 1993). Additionally, reducing the temperature to 15°C is necessary to initiate fruiting (Sakamoto et al., 2002).

Occasionally, the cultivated form can be found in the wild as well. Wild *F. velutipes* grows in this form when the mushrooms start growing under bark. There are no air currents underneath bark, so releasing spores underneath bark will prevent spore distribution. The cultivated form helps *F. velutipes* access air currents even when sporocarp formation begins under bark: the stipe gets much longer than normal to try and grow out from underneath the bark. While the mushroom is still under the bark, most resources are diverted to stipe growth, so the cap, gills, and colors do not develop. Once the mushroom reaches the top of the bark, it switches to growing in the normal form. High carbon dioxide occurs under bark because the mushroom is respiring but, with no
air flow, the carbon dioxide remains trapped under the bark. Bark also blocks incoming light, leading to low light conditions. The cultivation conditions that mushroom growers use to achieve the cultivated form mimic the conditions found under bark.

**The *F. velutipes* Genome**

The nuclear and mitochondrial genomes of *F. velutipes* have both been sequenced. Yoon et al. (2012) sequenced the mitochondrial genome of the monokaryotic strain *F. velutipes* 4019-20 from the Rural Development Administration of South Korea. The mitochondrial genome contained 15 protein-encoding genes and 26 tRNA-encoding genes. Park et al. (2014) sequenced the nuclear and mitochondrial genome of the monokaryotic strain KACC42780 (identical to strain 4019-20) from the Korean Agricultural Culture Collection. This study predicted 12,218 genes across the 11 chromosomes of *F. velutipes* and predicted 18 protein-encoding mitochondrial genes. Kurata et al. (2016) sequenced the genome of strain TR19, which they created by mating monokaryotic cultures from two strains used commercially in Japan. These researchers predicted the genome contained 13,843 genes. The sequence results of these three studies are available in the National Center for Biotechnology Information (NCBI) Genome database under ID number 16873. Additionally, researchers from the Fujian Agriculture and Forestry University submitted a genome of *F. velutipes* strain 6-3 to the NCBI Genome database in 2020 (GenBank assembly accession number GCA_011800155.1).

**Uses of *F. velutipes***

*F. velutipes* is most commonly used as an edible mushroom. It is widely cultivated, and the cultivated form is readily available in grocery stores. In the United States, it is primarily sold as “enoki” or “enokitake” in Asian groceries. As of 2010, over
two million metric tons of enoki were produced annually (Royse, 2014). The mushroom is also sought after in the wild by mushroom hunters, who collect and eat the normal form of the mushroom.

There is also some commercial interest in *F. velutipes* for its ability to degrade lignin. Lignin degrading enzymes are of commercial interest for their use in biopulping. This process uses enzymes to remove lignin from wood while leaving the valuable cellulose without having to use harsh chemicals. A major focus of the Park et al. (2014) study (which assembled the genome of *F. velutipes*) was in identifying genes related to lignin degradation.

*Flammulina velutipes* has also been used as a model organism for studying mushroom development. Developmental studies of *F. velutipes* mushrooms have largely focused on stipe elongation (for example, Gruen, 1969), which is particularly important in the cultivated form of the mushroom. However, *F. velutipes* has also been used to investigate fruiting induction; one study found that an extract from *F. velutipes* could induce fruiting (Urayama, 1969, as cited in Moore, 1998, p. 272). *Flammulina velutipes* has also been grown in space, confirming that fungi use gravity to orient themselves (Kern & Hock, 1993).

**Gene Expression in *F. velutipes* Development**

More recent studies using *F. velutipes* as a model organism have focused on how protein expression changes during mushroom development. What proteins a cell has ultimately determines its behavior, but there are multiple ways to study protein expression. The most direct way to assess protein expression is to look for the proteins themselves, but this is difficult and expensive, often resulting in small and not very
informative data sets. Consequently, most studies have examined gene expression instead, which involves the comparatively easy process of sequencing mRNA. In either case, the goal is to determine what events are happening inside the cells.

The first researchers to study gene expression in *F. velutipes* mushrooms were Azuma et al. (1996, as cited in Ando et al., 2001), who identified the gene *fds* and demonstrated that it was specifically expressed during mushroom development. The same group of researchers later identified the genes *fvfd16* and *fvfd30*, which were also expressed during fruitbody development (Ando et al., 2001; Kim et al., 1999; Kim & Azuma, 2000). Although these three genes were the first fruitbody-related genes to be characterized in *F. velutipes*, the function of all three is still unknown.

Soon after, Ando et al. (2001) identified the gene *fvh1*, which encoded for a hydrophobin and was specifically expressed in the mycelium after fruitbody initiation. Since then, hydrophobins have been a popular research target. Yamada et al. (2005) identified the hydrophobin gene *fv-hyd1* and found it was specifically expressed in young and mature fruitbodies. Sakamoto et al. (2007) discovered the hydrophobin PSH, which was specifically expressed in the pileus after light exposure. These hydrophobins and others routinely appear in later studies that assess multiple genes at once.

Sakamoto et al. (2002) were the first to examine expression patterns of multiple proteins at the same time. They extracted proteins from the mycelium, cultivated mushrooms, and normal mushrooms. After performing two-dimensional gel electrophoresis, visualizing the results, and comparing the protein spots between the three tissues, they identified 22 blots that were either missing from or were a different size than in the mycelium samples. This result demonstrated that protein expression did change
during mushroom formation in *F. velutipes*.

Yamada et al. (2006) identified 600 genes that were expressed in the primordium but not in the mycelium. Of those differentially expressed genes, only 29 could be identified. Expression patterns for those genes were assessed using PCR and gel electrophoresis, but because of the small sample size, the researchers could not draw conclusions about developmental events.

To get a better idea of what was happening inside the cells, Joh et al. (2009) sequenced 3,332 genes from the mycelium, primordium, and cultivated mushrooms. Of these genes, 28.5% were involved in biological processes, 27.7% had a molecular function, 5.9% coded for cellular components, and the remaining 37% had an unknown function. Their data also showed large differences in expression patterns among the three stages of growth: 994 of the genes were expressed only in the mycelium, 607 were expressed only in the primordia, and 1,088 appeared only in the cultivated form mushrooms.

Different results were obtained by Park et al. (2014) using RNA-seq. Those researchers compared 11,188 genes (representing the vast majority of the predicted 12,218 genes in *F. velutipes*) across the mycelia, primordia, and cultivated mushrooms and found that 10,121 genes were expressed in all three stages. Only 52 genes were unique to the mycelium, only 66 genes were unique to the primordium, and only 222 genes were unique to the mushrooms. Additionally, Park et al. (2014) examined the stipe and pileus tissue of the cultivated mushrooms and found 122 genes expressed in the stipe but not the pileus while 329 genes were expressed in the pileus but not the stipe.

Four further studies used RNA-seq to assess gene expression, but produced more
limited results. Liu et al. (2015) used the technique to assess expression of homocitrate synthase (Fvhcs), a gene involved in lysine biosynthesis. That gene was expressed at somewhat higher levels in the pileus than in the stipe, but the authors drew no conclusions about the gene’s impact on development. Wang et al. (2015) used RNA-seq to look at the 11 laccase genes in *F. velutipes*. One of the laccases, lac4, was expressed more highly than all other laccases during mushroom formation. Wu et al. (2018) investigated genes involved with cold-induced fruiting by assembling the transcriptome without using a reference genome. Their work resulted in a transcriptome of 20,157 unigenes (unique sequences presumed to represent genes) – a number much higher than the estimated total number of genes in *F. velutipes* (between 12,000 and 14,000; Kurata et al., 2016; Park et al., 2014). This discrepancy is likely the result of splice variants, but the researchers did not discuss possible explanations. Differentially expressed genes identified by Wu et al. (2018) were primarily associated with metabolism but also included genes involved in two-component signaling systems, MAPK signaling, and calcium signaling. This result indicates that early mushroom formation involves complex signaling systems as well as metabolic changes. Wang et al. (2019) used RNA-seq to examine 13 ergosterol biosynthesis genes during mushroom formation. Compared to the mycelium, six genes were upregulated and seven were downregulated in the young mushrooms. Compared to the young mushrooms, one gene was downregulated in the cultivated form mushrooms. Wang et al. (2019) drew no conclusions about how differential expression of ergosterol biosynthesis genes impacts fruitbody development.

The most thorough analysis of expression data was performed by Liu et al. (2017), who sequenced 1,198 proteins from the mycelium, primordia, and young
mushrooms. By matching the 171 differentially expressed proteins to known proteins in the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) databases, the researchers were able to determine which kinds of cellular components, biological processes, and molecular functions were upregulated. Many of the genes upregulated in the primordium and mushrooms were related to energy metabolism, suggesting that mushroom formation requires high levels of energy production. Genes that were upregulated in only the primordium were involved in the citric acid cycle, nucleotide synthesis, fatty acid synthesis, detoxification, pigment formation, and carbohydrate metabolism. Proteins involved in amino acid synthesis were also upregulated in primordia and mushrooms. In particular, proteins that synthesize branched-chain amino acids and aromatic amino acids were upregulated in the primordia, suggesting those types of amino acids could play a role early in mushroom development.

In recent years, researchers have begun to test what happens when gene expression in *F. velutipes* is artificially manipulated. The first such experiment was conducted by Lu et al. (2016), who tested the impact of over- or under-expressing *Fv-JRL1*, a jacalin-related lectin gene. In wild type cultures, *Fv-JRL1* was highly expressed during primordium formation. Reducing the expression levels using RNA interference (RNAi) increased the amount of time required for the mycelium to begin forming primordia, while overexpression transformations decreased the time to primordia formation. No other genes were analyzed by Lu et al. (2016) and the molecular function of *Fv-JRL1* was not determined.

Wu et al. (2019) used RNAi to test what happens when the gene *pdd1* is expressed at lower levels. When transcription of *pdd1* (a gene that contains an HMG-box
DNA-binding domain and a nuclear localization signal) was reduced by 89.9%, primordia failed to form. Analysis revealed that PDD1 positively regulated pheromone receptors, jacalin-related lectin encoding genes, and *fvfd16*. Overexpression of *pdd1* resulted in increased yield (by weight) of *F. velutipes* mushrooms by 33%, but gene expression in the fully grown mushrooms was not assessed.

The same researchers also studied knockdowns of the gene *Fvcpc2*, a CPC-2 gene containing seven WD-40 domains (Wu et al., 2020). Knocking down *Fvcpc2* prevented fruitbody formation and slowed vegetative growth. Vegetative growth was restored to wild type levels by addition of cAMP, suggesting that *Fvcpc2* participates in cAMP signaling pathways. The *Fvcpc2* knockdown also decreased levels of lectin encoding genes, hydrophobins, *Fvfd16*, and *Fvfd30*. Overexpression of *Fvcpc2* resulted in primordia forming earlier than in the wild type strain and increased mushroom yields (by weight) by 6.57%, but gene expression in the fully grown mushrooms was not assessed.

**Knowledge Gaps**

Previous studies into gene activity during *F. velutipes* fruitbody formation have provided detailed but incomplete information on a diverse array of genes. Researchers have largely focused on the early stages of mushroom growth and the cultivated growth form (Table 1). Although six studies grew mushrooms in the normal form, only four genes expressed in the normal form have ever been identified and analyzed for expression. Furthermore, only four studies have assessed gene activity at the tissue level and none of those considered gill tissue, tissues within the primordia, or tissues in mature normal mushrooms. To complete the picture of gene expression in *F. velutipes*, a study needs to be done that assesses expression levels of all genes in all tissues at all developmental
Table 1. Summary of research into gene or protein expression in *Flammulina velutipes*.

<table>
<thead>
<tr>
<th>Paper</th>
<th>Mycelium*</th>
<th>Primordium*</th>
<th>Young Mushroom*</th>
<th>Mature Cultivated*</th>
<th>Cultivated + Light*</th>
<th>Mature Normal*</th>
<th>Total Genes **</th>
<th>Genes Analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kim et al., 1999&lt;sup&gt;###&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>1 (fyfd30)</td>
<td></td>
</tr>
<tr>
<td>Ando et al., 2001&lt;sup&gt;###&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>1 (fyh1)</td>
<td></td>
</tr>
<tr>
<td>Sakamoto et al., 2002&lt;sup&gt;###&lt;/sup&gt;</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>?</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Yamada et al., 2005&lt;sup&gt;###&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>1 (fy-hyd1)</td>
<td></td>
</tr>
<tr>
<td>Yamada et al., 2006</td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>600</td>
<td>29</td>
</tr>
<tr>
<td>Sakamoto et al., 2007</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>Stipe Pileus</td>
<td>10</td>
</tr>
<tr>
<td>Joh et al., 2009</td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3,332</td>
<td>3,332</td>
</tr>
<tr>
<td>Park et al., 2014</td>
<td></td>
<td></td>
<td>-</td>
<td>Stipe Pileus</td>
<td>-</td>
<td>-</td>
<td>All</td>
<td>11,188</td>
</tr>
<tr>
<td>Liu et al., 2015</td>
<td></td>
<td>-</td>
<td>Stipe Pileus</td>
<td>Stipe Pileus</td>
<td>-</td>
<td>-</td>
<td>All</td>
<td>1 (fvcys)</td>
</tr>
<tr>
<td>Wang et al., 2015</td>
<td></td>
<td></td>
<td>Stipe Pileus</td>
<td>Stipe Pileus</td>
<td>-</td>
<td>-</td>
<td>All</td>
<td>11 (laccases)</td>
</tr>
<tr>
<td>Lu et al., 2016</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1 (Fv-JRL1)</td>
<td></td>
</tr>
<tr>
<td>Liu et al., 2017</td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1,198</td>
<td>171</td>
</tr>
<tr>
<td>Wu et al., 2018</td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>All</td>
<td>20,157</td>
</tr>
<tr>
<td>Wu et al., 2019</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>1 (pdd1)</td>
<td>794</td>
</tr>
<tr>
<td>Wang et al., 2019</td>
<td></td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>All</td>
<td>14 (ergosterol biosynthesis)</td>
</tr>
<tr>
<td>Wu et al., 2020</td>
<td></td>
<td>-</td>
<td>Pheno</td>
<td>-</td>
<td>-</td>
<td>Pheno</td>
<td>1</td>
<td>1 (Fvcep2)</td>
</tr>
</tbody>
</table>

*Symbols used: check mark (✔) means the stage was assessed, dash (-) means the stage was not assessed, and “Pheno” means only phenotypic data was reported. When tissues were examined, the tissues are listed instead of a check mark. A question mark indicates the information could not be determined.

***“All” indicates an RNA-Seq study was performed to sequence all the mRNA in the samples. A question mark indicates the information could not be determined.

<sup>#</sup>As cited in Ando et al., 2001.

<sup>###</sup>Studies were conducted as a time course with timepoints beginning just before fruitbody initiation and ending at mature spore-producing mushrooms.
One additional problem with previous research into *F. velutipes* is that each study has its own definitions for the various stages. For example, “mature” in some studies refers to the cultivated form but other studies use the term to mean the normal form. Taking all previous studies together, there are five stages that can be readily identified for comparison between studies: the mycelium, primordia, young mushrooms, mature cultivated mushrooms, and mature normal mushrooms. The mycelium is just a network of hyphae not incorporated into a fruitbody. Previous work has assessed the mycelium before fruiting initiation, but has largely neglected the mycelium present during mushroom development: only one gene, *fvh1*, has been studied in the mycelium after fruitbody formation (Ando et al., 2001). The primordia are small structures with stipe, pileus, and gill tissue but without an elongated stipe. Young mushrooms have an elongated stipe but the pileus margin is still attached to the stipe. This stage is present both when grown in darkness with high CO₂ (to produce cultivated mushrooms) and when grown in light with ambient CO₂ (to produce the normal mushrooms). Previous studies refer to this stage variously as the “elongation” stage, the “young mushroom” stage, or the “pinhead fruitbody” stage. Mature cultivated mushrooms have a long stipe, small pileus, and exposed gill tissue (the pileus margin has detached from the stipe) and are produced by growing the mushrooms in low light with a high concentration of CO₂. Previous research uses several terms for this stage, usually either “maturation” or “pinhead fruitbody.” Mature normal mushrooms are easily differentiated by the fact that they produce copious spores from the gills. Additionally, the gills and pileus are well developed, the pileus is yellow to orange, and the stipe is yellow to black. This stage is
usually referred to as either the “maturation” or “sporulation” stage and is produced when the mushrooms are grown under light with an ambient CO2 concentration. Although all five stages have been researched previously, no single study has analyzed all five stages at once. Because of this and because of inconsistent definitions, it is difficult to compare the results of different studies. *Flammulina velutipes* developmental research would therefore greatly benefit from a study assessing all five developmental stages.

A study considering all five stages would also help mycologists to design experiments that manipulate specific genes. Although some researchers have already begun manipulating fruiting-related genes in *F. velutipes*, those studies have been limited to studying the early stages of fruiting. Knockdowns that prevent fruitbody formation make it hard to research what happens in mature mushrooms. Having detailed expression data for wild-type mature mushrooms would benefit manipulative studies by providing a baseline.

Even though previous studies are limited, they have provided useful background information by identifying many genes of interest that should be considered in future research. Three genes are particularly interesting because they have an unknown function but are repeatedly found to be upregulated during mushroom development: *fds*, *fvfd16*, and *fvfd30* (Ando et al., 2001; Joh et al., 2009; Kim & Azuma, 2000; Liu et al., 2017; Park et al., 2014). Other interesting genes are those known to be important parts of common cellular networks, including cytochrome P450s, MAPK signaling pathway genes, and calcium signaling genes (Liu et al., 2017; Wu et al., 2018; Yamada et al., 2006). Several studies report that hydrophobins are differentially expressed during development (Ando et al., 2001; Joh et al., 2009; Liu et al., 2017; Park et al., 2014;
Sakamoto et al., 2007; Yamada et al., 2005), making them useful targets for assessing cell differentiation. Metabolic genes, elongation factor 1-alpha, and the protease pathway are mentioned in multiple studies and should also be examined in greater detail (Joh et al., 2009; Liu et al., 2017; Wu et al., 2018). Additionally, three genes have been the subject of knockdown studies, but their wild-type activity in all tissues and stages has yet to be fully assessed: Fv-JRL1, pdd1, and Fvcpc2 (Lu et al., 2016; Wu et al., 2019, 2020).

**Objectives of the Current Study**

1) Identify differences in gene expression between the three major tissues of *F. velutipes*. Tissue-level differences in gene expression will help describe how the different mushroom tissues are differentiated from one another. Additionally, this will complement previous studies by assessing expression in primordial tissues and gill tissue, which have not been studied before.

2) Identify differences in gene expression within each *F. velutipes* tissue (stipe, pileus, and gills) as development progresses. These differences will help identify genetic pathways related to tissue differentiation.

3) Identify differences in gene expression between the normal and cultivated forms of *F. velutipes*. These differences will help identify genetic pathways related to tissue differentiation and possibly environmental sensing. Examining both forms will also enable comparisons with previous studies that considered only one of the forms.
CHAPTER II

RNA-SEQ REVEALS TISSUE-SPECIFIC AND GROWTH-RELATED GENE EXPRESSION IN FLAMMULINA VELUTIPES MUSHROOM DEVELOPMENT

Introduction

*Flammulina velutipes* is a widely cultivated mushroom sold commercially as “enoki” or “enokitake.” In the wild, *F. velutipes* grows as an umbrella-shaped mushroom with a glutinous orange pileus and velutinous black stipe. When cultivated, however, the mushrooms have a long pale stipe, tiny pale pileus, and underdeveloped gills. The cultivated form of the mushroom is also seen in the wild where sporocarps form under bark (a high CO₂ and low light environment). By devoting resources to forming a long stipe, the mushrooms increase their chances of finding a crack in the bark through which they can access fresh air for efficient spore release.

Because of the unusually long stipe in cultivated mushrooms, *F. velutipes* is also used to study sporocarp growth during development. Mushroom growth can either be determinate or indeterminate. Determinate growth is recognized when sporocarps push aside debris and nearby mushrooms, whereas indeterminate growth is recognized when sporocarps engulf debris and fuse with nearby mushrooms. *Flammulina velutipes*, along with other typical gilled mushrooms, exhibit determinate growth. Researchers have studied *F. velutipes* to understand the molecular controls governing this type of growth, from hormones to cell signaling pathways (Gruen, 1969; Liu et al., 2017). Most recently,
researchers have turned to gene expression analysis to understand the changes taking place during development (Lu et al., 2016; Park et al., 2014; Wu et al., 2018, 2019).

Gene expression has been studied in five major stages of *F. velutipes* mushroom growth: mycelium, primordia, young mushrooms, mature cultivated mushrooms, and mature normal mushrooms (Chapter I, Table 1). The mycelium is the most frequently studied stage in previous reports and consists of a loose network of assimilative hyphae that represents the bulk of the individual fungus (Liu et al., 2017). Although most research focuses on the mycelium before fruitbody initiation, the assimilative mycelium is still present and active during mushroom production (Ando et al., 2001). The primordium is the first stage of mushroom formation with a distinct cap and stipe. *Flammulina velutipes* primordia are shaped like rounded triangles with the pileus at the tip of the triangle (Liu et al., 2017). Young mushrooms are distinguished from earlier stages by their elongated stipe and from later stages by having a pileus margin still attached to the stipe (Liu et al., 2017; Sakamoto, 2010). Mature cultivated mushrooms are grown in the dark with a high CO$_2$ concentration, which mimics conditions under bark and results in an elongated stipe, small pileus, underdeveloped gills, and pale colors (Park et al., 2014; Sakamoto et al., 2002). Mature normal mushrooms are grown in light with an ambient CO$_2$ concentration, form a large circular yellow to orange pileus with well-developed gills that release spores, and develop a dark fuzzy stipe (Kim et al., 1999; Sakamoto et al., 2002; Wu et al., 2019).

Previous research has identified several genes that are differentially expressed in one or more of those stages. Multiple studies confirm that *fds*, *fyfd16*, and *fyfd30* are specifically expressed in the fruitbody, but their function or developmental role is
unknown (Ando et al., 2001; Joh et al., 2009; Kim & Azuma, 2000; Liu et al., 2017; Park et al., 2014). Cytochrome P450 enzymes, the mitogen-activated protein kinase (MAPK) signaling pathway, and the calcium signaling pathways, are also known to be more highly expressed in mushrooms than in undifferentiated mycelium (Liu et al., 2017; Wu et al., 2018; Yamada et al., 2006). Additionally, hydrophobin genes exhibit tissue-specific expression, with *psh* expressed in the pileus and *fvh1* expressed in the mycelium after fruiting initiation (Ando et al., 2001; Joh et al., 2009; Liu et al., 2017; Park et al., 2014; Sakamoto et al., 2007; Yamada et al., 2005). Mushrooms also undergo metabolic changes during mushroom formation, increasing expression of genes needed to produce and recycle cellular components (Joh et al., 2009; Liu et al., 2017; Wu et al., 2018). Recently, knockdown studies have been used to investigate the impacts of a few specific genes. Reducing transcript abundance of *Fv-JRL1* by 26% and 51% using RNA interference (RNAi) resulted in slower assimilative mycelial growth and delayed primordium formation (Lu et al., 2016). Similarly, when *pdd1* transcripts were reduced by 30.2% – 89.9%, mycelial growth slowed, primordial formation was delayed, and fewer mushrooms were produced (Wu et al., 2019). Reducing *Fvcpc2* transcripts by 67.7% – 69.7% also decreased mycelial growth, but completely blocked sporocarp formation (Wu et al., 2020).

Previous studies of gene activity during *F. velutipes* fruitbody formation have provided detailed yet incomplete information on a diverse array of genes. Researchers have largely focused on the early stages of mushroom growth and the cultivated growth form (Chapter I, Table 1). Although six studies grew mushrooms in the mature normal form, only four genes expressed in that stage have been identified and their expression
analyzed. Furthermore, only four studies have assessed gene activity at the tissue level, and none of those examined gill tissue, specific tissues within the primordia, or tissues in mature normal mushrooms. Completing the picture of gene expression in *F. velutipes* requires assessing expression levels of all genes in all tissues at all developmental stages.

The present study used RNA-seq to examine gene expression patterns in the stipe, pileus, gills, and mycelium collected from mushrooms at all major growth stages. This detailed picture of gene expression in *F. velutipes* fruitbody revealed that tissue has a large impact on gene expression differences. Furthermore, genes previously associated with mushroom development tended to be most highly expressed in actively growing tissues, suggesting that cell growth regulation is a major process underlying mushroom development.

**Materials and Methods**

**Cultivation, Fruiting, and Sampling**

A new strain of *Flammulina velutipes* was developed by culturing a wild mushroom sample collected in La Crosse, WI (43.814533°N, 91.205902°W) on 20 October 2019. The source mushroom was morphologically identified as *F. velutipes* (Appendix A, Figure 11) and is recorded on iNaturalist at https://www.inaturalist.org/observations/35015296. A tissue sample was taken from the mushroom and cultured on malt extract agar before being transferred to and maintained on potato dextrose agar (PDA). Both media were purchased from Difco and supplemented with 100 µg/ml streptomycin sulfate.

To create the spawn, fully colonized PDA plates were cut into small pieces using a sterilized scalpel and added to sterilized glass quart jars containing 276 g rye grain and
175 g water (Stamets, 1993). The jars were placed in an incubator at 25°C until fully colonized.

The fruiting substrate consisted of 76 g sawdust (34% mixed hardwood sawdust, 66% aspen shavings), 21 g rye grain, and 175 g water (Stamets, 1993). Glass quart jars containing the fruiting substrate were sterilized by autoclaving, cooled, and then the remaining space in the jar was filled with colonized spawn. The fruiting jars were shaken to evenly distribute the spawn and then placed in an incubator at 25°C until fully colonized.

The fruiting jars were then transferred to a fruiting chamber that maintained a temperature between 13°C and 18°C to induce fruiting (Sakamoto et al., 2002; Stamets, 1993). An LED light bulb (9.5 W, 120 V, 800 lumens, color profile of 2700 K) provided constant white overhead light (Sakamoto et al., 2007). For each batch, four jars were placed in the chamber at once. Each jar was randomly assigned a position in the chamber and randomly assigned one of four growth stages (primordial, young mushroom, mature cultivated, mature normal) such that each batch included one jar for each stage. Once primordia formed, a cover was placed over the jar assigned to the cultivated stage in order to increase CO₂ concentration and block light (Stamets, 1993).

Samples were collected from a jar once mushrooms in that jar reached the assigned developmental stage (Appendix A, Figure 12). Mushroom stages were easily distinguished from each other by the ratio of the pileus to the stipe (Appendix A, Figure 13). Tissue samples were cut from the stipe, pileus, and gills of each mushroom. Additionally, a sample was collected from the internal mycelium of each jar. Once each tissue was sampled, it was immediately frozen in liquid nitrogen and stored separately at
-20°C and later transferred to -80°C for long-term storage.

The entire process was repeated three times, resulting in a total of 48 samples: four developmental stages (primordial, young, mature cultivated, and mature normal) with four tissues of each (mycelium, stipe, pileus, and gills) and three replicates of each tissue. Although multiple mushrooms were collected from each jar, only one sample of each tissue from each jar was carried forward (Appendix A, Table 2).

**mRNA Extraction and Sequencing**

Total RNA extraction was completed with the Invitrogen PureLink™ RNA Mini Kit and each sample was eluted in 50 mL of sterile distilled water. RNA was quantified and quality was assessed on a Bio-Rad Experion instrument using Std Sense RNA chips. Quantification was confirmed on a subset of samples using a ThermoFisher NanoDrop spectrophotometer. Samples above 12 ng/µL RNA were diluted to 12 ng/µL and 25 mL of each sample was used for library preparation. A library was prepared using the KAPA HyperPrep mRNA Kit and Adapterama primers (Glenn et al., 2019), following the kit directions but using half the specified volumes (Oladeinde et al., 2018). Each sample received a unique index sequence in both the forward and reverse directions (Appendix A, Table 2). Individual libraries were pooled, and the pooled library was condensed to a 50 mL volume using a Savant SpeedVac vacuum concentrator. The library was submitted to the University of Wisconsin-Madison Biotechnology Center DNA Sequencing Facility, where sequencing was performed using the Illumina NovaSeq 6000 platform.

**Sequence Analysis**

Primer sequences, adapter sequences, and low-quality reads were removed from the demultiplexed sequence data using Trimmomatic version 0.38 (sliding window of 4
with a minimum quality score of 20; minimum length 50 base pairs; Bolger et al., 2014), and low-complexity sequences were removed using FQTrim version 0.9.7 (option -D; minimum length 30 base pairs; Pertea, 2018).

After initial sequence processing, analysis followed the New Tuxedo pathway (Pertea et al., 2016). Paired and unpaired reads were mapped to the reference genome (GenBank assembly accession number GCA_011800155.1) using HISAT2 version 2.2.1 (Kim et al., 2019). StringTie version 1.3.6 (Pertea et al., 2015) was used to assemble transcripts for each sample, assemble the complete transcriptome, and estimate read abundance for each sample, expressed as fragments per kilobase of transcript per million mapped reads (FPKM). Transcriptome completeness was assessed using BUSCO version 4.1.4 (Manni et al., 2021) to compare against the agaricales_odb10 database.

All transcripts in the transcriptome were searched against fungal 18S rRNA, 28S rRNA, and ITS databases from the NCBI RefSeq Targeted Loci Project (ftp://ftp.ncbi.nlm.nih.gov/refseq/TargetedLoci/Fungi/; Schoch et al., 2014) using BLASTN from the NCBI BLAST+ suite, version 2.10.0 (Camacho et al., 2009). Transcripts matching fungal rRNA were removed from the expression tables. Only samples containing transcript data for at least 300 unique non-RNA genes were used for differential expression analysis (Appendix A, Table 2 & Table 3).

Principal components analysis (PCA) was conducted using the R statistical computing environment (R version 4.1.0) to test the effect each sample had on expression patterns. PCA plots were generated to compare the effects of growth stage and tissue as well as the potentially confounding effects of batch, jar, and position.

Ballgown version 2.26.0 (Frazee et al., 2015), a Bioconductor package for R, was
used to identify differentially expressed genes, defined as having a \( q \)-value (a significance measure based on false discovery rate; Storey & Tibshirani, 2003) less than 0.05. For most analyses, tissue was specified as a covariate and developmental stage as a confounding variable. For pairwise comparisons between growth stages, stage was set as a covariate and tissue as a confounding variable. Differentially expressed genes were annotated using BLASTX from the BLAST+ suite to search the UniProtKB Agaricales database (taxon ID 5338). Heatmaps were generated in R with data transformed by \( \log_2(\text{FPKM}+1) \) and using the default clustering settings.

The pipeline used for data analysis is available at https://github.com/roehl-t/enoki. HISAT2, StringTie, and Ballgown were run with the settings suggested by Pertea et al. (2016).

Results

Sequence Data

The demultiplexed data consisted of 183,790 Mbases of paired-end sequences (543.6 million individual reads, 11.3 million reads/sample ± 12.0 million reads/sample standard deviation). After trimming, 473.3 million reads remained, with an average of 9.9 million reads per sample (standard deviation 9.5 million reads).

Transcriptome Assembly

The assembled transcriptome consisted of 20,909 transcripts from 14,416 potential genes. *Flammulina velutipes* is predicted to have around 13,843 genes in its genome (Kurata et al., 2016), which suggests the transcriptome covers the vast majority of the *F. velutipes* genome but some sequences identified as genes in the analysis are likely splice variants. A similar result was obtained through BUSCO analysis, which
revealed that the transcriptome covered 71.8% of core Agaricales genes (71.8% core genes included and complete, 9.1% included but fragmented, 19.1% missing, 3,870 core reference genes searched).

**Principal Components Analysis**

Plots generated by principal components analysis revealed that samples did not cluster by batch, jar, or position in fruiting chamber, demonstrating that batch effects did not influence transcription (Appendix B, Figures 14, 15). However, samples did cluster by tissue and weakly clustered by growth stage (Appendix B, Figure 16). The first two principal components explained a combined 64.7% of the total variance (Appendix B, Table 4).

**Differential Gene Expression**

Ballgown analysis identified 2,183 differentially expressed genes, 1,456 of which corresponded to named proteins in the UniProtKB database. All differentially expressed genes were plotted in a heatmap, which revealed one mycelial sample (46) with a different expression profile than all other samples (Figure 4). The rest of the mycelial samples had similar expression profiles to each other, with very low expression for all differentially expressed genes. Gill samples tended to cluster with the mycelium samples and all the normal mushroom gill samples clustered together with the mycelial samples. Stipe samples had very similar expression patterns to one another, but the remaining tissue samples did not cluster in a noticeable pattern.

To further analyze gene expression patterns, genes were divided into eight major clusters based on the heatmap (Figure 4). Gene expression of clusters 1, 2, and 4 was high in mushroom tissues (stipe, pileus, and gills), with the most highly expressed genes
grouped in cluster 1. Clusters 1, 2, and 4 included lectins and WD40 repeat-like proteins, which have previously been linked to development. The lectin \textit{Fv-JRL1} reduced primordium formation and slowed mycelial growth (Lu et al., 2016), while the seven-WD40 domain containing protein \textit{Fvcpc2} completely blocked primordium formation.

Figure 4. Tissue-level gene expression (log2(FPKM+1)) of \textit{F. velutipes} mushrooms at four developmental stages. Samples are labeled as sample number followed by growth stage and source tissue. Stage abbreviations: r = primordium, y = young mushrooms, c = mature cultivated, n = mature normal. Tissues abbreviations: m = mycelium, s = stipe, p = pileus, g = gills. Major clusters on the left-hand dendrogram are numbered 1-8.
(Wu et al., 2020). In the present study, one lectin appeared in cluster 1, while a second lectin and four WD40 repeat-like proteins grouped with cluster 4 (Appendix C, Figures 17, 18). Three additional WD40 repeat-like proteins were differentially expressed, one of which grouped with cluster 6 while the other two grouped with cluster 8 (Appendix C, Figure 18). A subset of genes in cluster 4 exhibited higher levels of gene expression in the gills and pileus (Appendix C, Figure 19). Stipe tissue had the most consistent gene expression and clusters 3 and 7 included genes that were most highly expressed in the stipe. Genes in cluster 5 showed particularly low expression in mushroom tissues, but only 10 of the 25 genes in were named in the UniProtKB database (Appendix C, figure 20). This cluster included the gene *pdd1*, a transcription factor that helps trigger primordium formation (Wu et al., 2019). Cluster 6 genes were highly expressed in sample 46 and expressed at medium levels in mushroom tissues. Cluster 8 genes were highly expressed only in sample 46. Of those genes, 50.2% either did not match any proteins in the UniProtKB database or matched uncharacterized genes or unplaced scaffolds.

The genes *fds* and *fvfd16* had similar expression patterns: they were more highly expressed in mushroom tissues than in mycelium and had their highest expression levels in stipe tissue (Figure 5). However, both genes were expressed at low levels in the primordial pileus, young mushroom gills, and normal mushroom gills. Both *fds* and *fvfd16* are consistently linked to *F. velutipes* mushroom development, although their functions are unknown (Ando et al., 2001; Kim & Azuma, 2000; Liu et al., 2017; Park et al., 2014).

Twenty differentially expressed cytochrome p450 genes were detected among the samples (Figure 6). Cytochrome p450 enzymes have previously been found to be more
highly expressed during fruitbody formation (Liu et al., 2017; Yamada et al., 2006). The cytochrome p450 matching UniProt accession A0A2H3CCC9 was most highly expressed in the pileus and gill tissues. The one matching UniProt accession A0A2H3BG08 was most highly expressed in stipe tissue. Four cytochrome p450 genes (matching A0A2H3D3X5, A0A2H3DB07, A0A2H3BPS0, and A0A2H3B3A6) appeared almost exclusively in stipe tissue and had the highest expression levels in cultivated or normal mushroom stipe tissue.

Three genes from the mitogen-activated protein kinase (MAPK) pathway were detected in the current study (Figure 7). The MAPK signaling pathway is used in many contexts and multiple studies have shown MAPK pathway expression increases during sporocarp development (Liu et al., 2017; Wu et al., 2018). One of those, a MAPK that matched UniProtKB accession A0A2H3CIG4, was strongly expressed in all mushroom

![Figure 5: Expression of fds and vfd16 in four Flammulina velutipes tissues at four developmental stages.](image)
Figure 6: Expression (log$_2$(FPKM+1)) of 20 cytochrome p450 genes in four *Flammulina velutipes* tissues at four developmental stages. Each gene is labeled with its matched protein name followed by the UniProtKB accession number in parentheses.
tissues, but was present at lower levels in the normal mushroom pileus and gills.

Only three hydrophobin genes were differentially expressed in the current study (Figure 8). Hydrophobins are small proteins that self-assemble into rodlet layers that coat the exterior of hyphae and are known from previous studies to have tissue-specific expression in *F. velutipes* (Ando et al., 2001; Sakamoto et al., 2007; Wösten et al., 1994). The hydrophobin matching accession A0A0D2NYY5 was highly expressed in all mushroom samples. However, the hydrophobin matching UniProtKB accession G8A517 was found only in stipe tissue and in sample 46.

Another gene expressed at high levels specifically in stipe tissue is a gene that

![Figure 7: Expression of three mitogen-activated protein kinase-related genes in four Flammulina velutipes tissues at four developmental stages. Each gene is labeled with its matched protein name followed by the UniProtKB accession number in parentheses.](image-url)
matched cell division cycle protein 123 (cdc123, UniProtKB accession A0A2H3BK13; Figure 9). This gene was detected in only one mycelium sample, expressed most highly in the stipe, and at low to moderate levels in the pileus and gills. In pileus tissue, cdc123 expression was variable, but tended to increase with sporocarp maturity. Gill tissue expression of cdc123 was also variable, but peaked in the cultivated mushrooms.

Four genes related to heat-shock proteins (HSPs) were differentially expressed. HSPs are important for protein folding and previous work found them to be upregulated in young mushrooms (Liu et al., 2017). All four differentially expressed HSPs in the current study were highly expressed in mushroom tissues during the primordial, young

![Figure 8: Expression of three hydrophobin genes in four Flammulina velutipes tissues at four developmental stages. Each gene is labeled with its matched protein name followed by the UniProtKB accession number in parentheses.](image)
mushroom, and cultivated stages, but expression decreased in the normal stage (Figure 10). Additionally, expression tended to be highest in the gill tissue, except in the normal stage.

No differentially expressed genes were identified by pairwise comparisons between the four growth stages.

**Discussion**

The effect of tissue on differential gene expression has largely been ignored by previous research into *F. velutipes* development. Additionally, relatively little effort has gone into examining gene expression in mature mushrooms. The present study closes those research gaps by reporting gene expression differences for all tissues in the primordium, young mushrooms, mature cultivated mushrooms, and mature normal stages.
mushrooms. Results confirmed that tissue-level differential gene expression is a major component of *F. velutipes* sporocarp development.

**Tissue-Specific Gene Expression of Cytochrome p450**

Some of the most intriguing genes involved in sporocarp development are those encoding cytochrome p450 proteins. In *Coprinopsis cinerea*, the *eln2* gene encodes a cytochrome p450 protein and mutations to that gene will prevent stipe elongation (Muraguchi & Kamada, 2000). The present work revealed 20 differentially expressed genes.

![Heat-shock protein expression](image)

**Figure 10:** Expression of five genes related to heat-shock proteins in four *Flammulina velutipes* tissues at four developmental stages. Each gene is labeled with its matched protein name followed by the UniProtKB accession number in parentheses.
genes encoding cytochrome p450 enzymes in *F. velutipes*, including four that were stipe-specific and a fifth which was most highly active in the stipe (Figure 6). Additionally, one cytochrome p450 gene was found to be specifically expressed in the pileus and gills. This work adds to the evidence that cytochrome p450 expression is linked to fruitbody development in *F. velutipes* but further suggests that different cytochrome p450 genes are used to control development in different tissues. The family of cytochrome p450 genes could therefore play a key role in regulating mushroom growth and also be a major factor in tissue differentiation. This observation should be tested by experimentally manipulating these six cytochrome p450 genes in *F. velutipes* and other model organisms.

**A Stipe-Specific Hydrophobin**

A few hydrophobins have been identified in *F. velutipes*, with at least two showing tissue-specific expression: FVH1 is expressed in the mycelium after sporocarp initiation, while PSH is expressed in the pileus after light exposure (Ando et al., 2001; Sakamoto et al., 2007). Three hydrophobins were detected in the current study and all were more highly expressed in mushroom tissues than in the mycelium (Figure 8). However, only one (UniProtKB accession G8A517) showed tissue-specific expression. This hydrophobin is the first reported to be stipe-specific in *F. velutipes*. Hydrophobin G8A517 adds a third known tissue-specific hydrophobin to the *F. velutipes* system. This suite of tissue-specific hydrophobins could allow future research to use hydrophobins as markers for tissue differentiation.
Increased Expression in Actively Growing Tissues

Genes that were previously linked to sporocarp development in *F. velutipes* tended to be most highly expressed in actively growing tissue in the current study. Two of the earliest genes to be recognized as differentially expressed during mushroom formation were *fds* and *fvfd16* (Ando et al., 2001; Kim & Azuma, 2000). Although these genes have consistently been linked to mushroom development in *F. velutipes*, their functions and mechanisms of action are unknown. In the present work, both genes exhibited highest activity in the stipe and lowest activity in the mature normal pileus and gills (Figure 5).

The expression patterns of MAPK A0A2H3CIG4, hydrophobin A0A0D2NYY5, and HSPs are likewise lowest in the normal pileus and gills (Figures 7, 8, 10). Previous research has demonstrated that the MAPK signaling pathway is highly expressed during *F. velutipes* mushroom development (Liu et al., 2017; Wu et al., 2018). Likewise, *F. velutipes* hydrophobins that are not tissue-specific are typically highly expressed throughout the sporocarp (Yamada et al., 2005; Park et al., 2014). Previous research on HSPs in *F. velutipes* has suggested they are highly expressed in young mushrooms and are possibly important for triggering fruiting under reduced temperature (Liu et al., 2017; Wu et al., 2018). Results from the present study indicate that MAPKs, hydrophobins, and HSPs follow the same pattern as *fds* and *fvfd16*: the genes are important throughout development, but their expression levels decrease in tissues that are not actively growing.

Growth is most evident in the *F. velutipes* stipe as it rapidly elongates, but slows as the mushroom reaches its full size (Gruen, 1969). Growth-related genes are therefore expected to be most highly expressed in the stipe and in developing tissues before the
mature normal stage. Because fds, fvd16, MAPK genes, the hydrophobin matching A0A0D2NNY5, and HSPs follow the expected growth-related pattern, they may be involved in cell growth regulation. Cell growth is the primary driver of mushroom growth and morphogenesis (Hammad et al., 1993; Moore et al., 1979); regulating cell growth may therefore be the primary mechanism for controlling mushroom growth after primordium formation.

Two additional gene groups had similarly low expression in the mature normal pileus and gills: lectins and WD40 repeat-containing genes (Appendix C, Figures 17, 18). In previous studies, knocking down Fv-JRL1 (a jacalin-related lectin) or fvcpc2 (a CPC-2 encoding gene with seven WD40 repeats) resulted in reduced primordium formation but also slowed mycelium growth (Lu et al., 2016; Wu et al., 2019, 2020). Slower mycelial growth would be expected if the same genes that drive mushroom growth also have a role in cell growth throughout the mycelium.

One such gene could be cdc123, which was revealed by this study to be particularly active in stipe tissue and had moderate expression in the gills and pileus with reduced expression in the mature normal gills (Figure 9). This gene is an essential cell cycle regulator that functions in nutritional control of START – the point in G1 where the cell cycle is arrested until cyclin-dependent kinase 1 triggers entry into S phase (Bieganowski et al., 2004). Additionally, cdc123 promotes assembly of the eukaryotic translation initiation factor 2 (eIF2) complex (Perzlmaier et al., 2013). The eIF2 complex carries methionyl-tRNA and is necessary to begin translation. Cdc123 is widely conserved in eukaryotes and the human homolog of cdc123 can functionally replace Saccharomyces cerevisiae cdc123 (Bieganowski et al., 2004; Perzlmaier et al., 2013). In
humans, *cdc123* is associated with basal breast cancer, type 2 diabetes, and reduced pulmonary function (Adélaïde et al., 2007; Artigas et al., 2011; Zeggini et al., 2008). Both its role as a cell cycle regulator and as a promoter of translation could play roles in driving the expression pattern of *cdc123* in *F. velutipes*. Since the expression of *cdc123* is highest in actively growing tissues, the gene’s function as a cell cycle regulator is particularly interesting. The *cdc123* gene could be an endpoint for cell growth regulation in the stipe and further efforts should be made to understand how *cdc123* activity and the rest of the cell cycle changes during mushroom development.

A third gene that was previously manipulated in a knockdown study, the transcription factor *pdd1*, was identified as a differentially expressed gene in the current study. In previous research, knocking down *pdd1* blocked primordium development, demonstrating that the gene is important early on in sporocarp formation (Wu et al., 2019). It grouped with genes in cluster 5, which were expressed at lower levels in the three mushroom tissues (Figure 4). Expression of *pdd1* was lowest in the gills and highest in the stipe, although the range of values in the mushroom tissues did not exceed the range of values in the mycelium (Appendix C, Figure 20). This lack of a clear pattern suggests that *pdd1* is not important for mushroom growth after the initial stages of development.

**No Differences in Stage Pairwise Comparisons**

One surprising result from this research is that growth stage was not as important for differential gene expression as was tissue specificity. Why were no differentially expressed genes detected between the growth stages even though there are large and readily observed phenotypic changes during mushroom development? One hypothesis is
that the regulatory changes underpinning the phenotypes happen very quickly. Alternatively, it could be that gene expression changes exist in smaller areas of the mushroom (such as the pileus-gill interface).

Further studies on *F. velutipes* development should consider more intensive sampling to catch rapid changes and/or should sample specific cell layers within tissues. Researchers should also consider studying tissue-level differences in the earliest stages of mushroom development in *F. velutipes*: the fruitbody initial and young primordium. The fruitbody initials in *F. velutipes* are small but form around an amber-colored droplet of liquid, making them easy to spot on a Petri dish. The early visual cues and occurrence of two distinct growth forms make *F. velutipes* an ideal model system for studying sporocarp development that should be advanced further.

**Sample 46**

Sample 46 had a unique expression pattern that was different from all other mycelium or mushroom samples (Figure 4). What caused this difference is unknown, but the jar from which the sample was collected possibly had mite or bacterial contamination. The genes highly expressed in sample 46 could therefore have previously unknown roles in immune responses and hyphal defenses. Over half of the genes expressed most abundantly in sample 46 were unknown, making them interesting targets for future research into secondary metabolites, drug discovery, and fungal immune responses.

**Implications**

Analysis of these tissue-level differences during *F. velutipes* mushroom growth revealed genes not previously reported as differentially expressed but also provided new details on most genes known to have a role in *F. velutipes* mushroom development. This
detailed picture of gene expression in *F. velutipes* will be useful for designing future research into mushroom development. Given how strong an impact tissue specificity has on gene expression, future work must be designed to account for tissue-level differences.

Furthermore, looking within tissues shows great promise for improving our current understanding of mushroom growth. Regulating cell growth appears to be the main mechanism fungi use to expand their sporocarps after primordium formation. The molecular pathways involved in cell growth should therefore be high-priority targets for future research into mushroom development. Cytochrome p450 genes and *cdc123* would be useful starting points to understand the interplay between cell growth and tissue differentiation, since both show tissue-specific expression and the former is known to play a role in mushroom formation while the latter is an integral part of the cell cycle. Elucidating the connections between these and other fruiting-related genes will help further characterize the molecular systems underpinning sporocarp development.
REFERENCES


Pertea, G. (2018). *Fqtrim: Filtering and trimming next generation sequencing reads* (0.9.7) [C++]. https://github.com/gpertea/fqtrim


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APPENDIX A

PHENOTYPIC DATA FOR MUSHROOMS SAMPLED
Figure 11. Morphological data used to identify the source mushroom as *Flammulina velutipes*. Collected in La Crosse, WI, USA (43.814533°N 91.205902°W) on October 20, 2019 from a hardwood log with bark attached (A). Pileus 1.3-3.5 cm, margin yellow to orange, center reddish orange to reddish brown, slimy (B). Stipe 1.2-2.5 cm, central, velvety, dark brown at the base, yellowish white at the apex (C). Gills yellowish white (C). Basidia four-sterigmate, 18 µm x 5 µm (D). Spores blunted ovals with a small apiculus and oil droplets, 6-7 µm x 2-3.5 µm (E). Recorded on iNaturalist at https://www.inaturalist.org/observations/35015296.
<table>
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<th>Primordial</th>
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<th>Mature Cultivated</th>
<th>Mature Normal</th>
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Figure 12. Photographic data of all mushrooms sampled, organized by batch and growth stage. Ruler in background shows cm (smallest tick marks are 1 mm).
Figure 13. Pileus width compared to stipe length for all mushrooms sampled (log$_{10}$ scale).
Table 2. Basic sample collection and processing information on all sequenced samples.

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<th>Reverse Primer</th>
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Table 3. Number of samples with 300 or more unique non-RNA transcripts, separated by stage and tissue.

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APPENDIX B

PRINCIPAL COMPONENTS ANALYSES
Figure 14. PCA plot of the effect of sample on transcription with samples distinguished by batch (dot shape) and jar (color).
Figure 15. PCA plot of the effect of sample on transcription with samples distinguished by position in fruiting chamber.
Figure 16. PCA plot of the effect of sample on transcription with samples distinguished by tissue (“tissue,” dot shape) and developmental stage (“type,” color).
Table 4. Principal components analysis statistics for the PCA plots in Figures 14-16.

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Figure 17. Expression of two lectin genes in four *Flammulina velutipes* tissues at four developmental stages. Each gene is labeled with its matched protein name followed by the UniProtKB accession number in parentheses.
Figure 18: Expression ($\log_2$(FPKM+1)) of seven WD40 repeat-like protein genes in four *Flammulina velutipes* tissues at four developmental stages. Each gene is labeled with its matched protein name followed in parentheses by the UniProtKB accession number and cluster designation from Figure 4.
Figure 19. Mean expression (log2(FPKM+1)) of differentially expressed genes in four tissues and four growth stages of *Flammulina velutipes*. The plotted genes are a subset from cluster 4 that are highly expressed in pileus and gill tissue (see Chapter II, Figure 4).
Figure 20. Expression of *pdd1* four *Flammulina velutipes* tissues at four developmental stages.